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(54) Title: METHODS AND COMPOSITIONS FOR POLYPEPTIDE ENGINEERING

(57) Abstract

Methods are provided for the evolution of proteins of industrial and pharmaceutical interest, including methods for effecting recombination and selection. Compositions produced by these methods are also disclosed.

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METHODS AND COMPOSITIONS FOR POLYPEPTIDE ENGINEERING

This application is a continuation-in-part of U.S. Patent Application Serial No. 08/198,431, filed February 17, 1994 (now U.S. Patent No. 5,605,793, issued February 25, 1997), Serial No. PCT/US95/02126, filed, February 17, 1995, Serial No. 08/425,684, filed April 18, 1995, Serial No. 08/537,874, filed October 30, 1995, Serial No. 08/564,955, filed November 30, 1995, Serial No. 08/621,859, filed March 25, 1996, Serial No. 08/621,430, filed March 25, 1996, Serial No. PCT/US96/05480, filed April 18, 1996, Serial No. 08/650,400, filed May 20, 1996, Serial No. 08/675,502, filed July 3, 1996, Serial No. 08/721,824, filed September 27, 1996, 08/722,660, filed September 27, 1996, and 08/769,062 filed December 18, 1996 the specifications of which are herein incorporated by reference in their entirety for all purposes.

Background of the Invention

Recursive sequence recombination entails performing iterative cycles of recombination and screening or selection to "evolve" individual genes, whole plasmids or viruses, multigene clusters, or even whole genomes (Stemmer, Bio/Technology 13:549-553 (1995)). Such techniques do not require the extensive analysis and computation required by conventional methods for polypeptide engineering. Recursive sequence recombination allows the recombination of large numbers of mutations in a minimum number of selection cycles, in contrast to traditional, pairwise recombination events.

Thus, recursive sequence recombination (RSR) techniques provide particular advantages in that they provide recombination between mutations in any or all of these, thereby providing a very fast way of exploring the manner in which different combinations of mutations can affect a desired result.

In some instances, however, structural and/or functional information is available which, although not required for recursive sequence recombination, provides opportunities for modification of the technique. In other instances, selection and/or screening of a large number of recombinants can be costly or time-consuming. A further problem can be the manipulation of large nucleic acid molecules. The instant invention addresses these issues and others.

Summary of the Invention

One aspect of the invention is a method for evolving a protein encoded by a DNA substrate molecule comprising:

(a) digesting at least a first and second DNA substrate molecule, wherein the at least a first and second substrate molecules differ from each other in at least one nucleotide, with a restriction endonuclease;

(b) ligating the mixture to generate a library of recombinant DNA molecules;

(c) screening or selecting the products of (b) for a desired property; and

(d) recovering a recombinant DNA substrate molecule encoding an evolved protein.

A further aspect of the invention is a method for evolving a protein encoded by a DNA substrate molecule by recombining at least a first and second DNA substrate molecule, wherein the at least a first and second substrate molecules differ from each other in at least one nucleotide and comprise defined segments, the method comprising:

(a) providing a set of oligonucleotide PCR primers, comprising at least one primer for each segment, wherein the primer sequence is complementary to at least one junction with another segment;

(b) amplifying the segments of the at least a first and second DNA substrate molecules with the primers of step (a) in a polymerase chain reaction;

(c) assembling the products of step (b) to generate a library of recombinant DNA substrate molecules;

(d) screening or selecting the products of (c) for a desired property; and

(e) recovering a recombinant DNA substrate molecule from (d) encoding an evolved protein.

A further aspect of the invention is a method of enriching a population of DNA fragments for mutant sequences comprising:

(a) denaturing and renaturing the population of fragments to generate a population of hybrid double-stranded fragments in which at least one double-stranded fragment comprises at least one base pair mismatch;

(b) fragmenting the products of (a) into fragments of about 20-100 bp;

(c) affinity-purifying fragments having a mismatch on an affinity matrix to generate a pool of DNA fragments enriched for mutant sequences; and

(d) assembling the products of (c) to generate a library of recombinant DNA substrate molecules.

A further aspect of the invention is a method for evolving a protein encoded by a DNA substrate molecule, by recombining at least a first and second DNA substrate molecule, wherein the at least a first and second substrate molecules share a region of sequence homology of about 10 to 100 base pairs and comprise defined segments, the method comprising:

(a) providing regions of homology in the at least a first and second DNA substrate molecules by inserting an intron sequence between at least two defined segments;

(b) fragmenting and recombining DNA substrate molecules of (a), wherein regions of homology are provided by the introns;

(c) screening or selecting the products of (b) for a desired property; and

(d) recovering a recombinant DNA substrate molecule from the products of (c) encoding an evolved protein.

A further aspect of the invention is a method for evolving a protein encoded by a DNA substrate molecule by recombining at least a first and second DNA substrate molecule, wherein the at least a first and second substrate molecules differ from each other in at least one nucleotide and comprise defined segments, the method comprising:

(a) providing a set of oligonucleotide PCR primers, wherein for each strand of each segment a pair of primers is provided, one member of each pair bridging the junction at one end of the segment and the other bridging the junction at the other end of the segment, with the terminal ends of the DNA molecule having as one member of the pair a generic primer, and wherein a set of primers is provided for each of the at least a first and second substrate molecules;

(b) amplifying the segments of the at least a first and second DNA substrate molecules with the primers of (a) in a polymerase chain reaction;

(c) assembling the products of (b) to generate a pool of recombinant DNA molecules;

(d) selecting or screening the products of (c) for a desired property; and

(e) recovering a recombinant DNA substrate molecule from the products of (d) encoding an evolved protein.

A further aspect of the invention is a method for optimizing expression of a protein by evolving the protein, wherein the protein is encoded by a DNA substrate molecule, comprising:

(a) providing a set of oligonucleotides, wherein each oligonucleotide comprises at least two regions complementary to the DNA molecule and at least one degenerate region, each degenerate region encoding a region of an amino acid sequence of the protein;

(b) assembling the set of oligonucleotides into a library of full length genes;

(c) expressing the products of (b) in a host cell;

(d) screening the products of (c) for improved expression of the protein; and

(e) recovering a recombinant DNA substrate molecule encoding an evolved protein from (d).

A further aspect of the invention is a method for optimizing expression of a protein encoded by a DNA substrate molecule by evolving the protein, wherein the DNA substrate molecule comprises at least one lac operator and a fusion of a DNA sequence encoding the protein with a DNA sequence encoding a lac headpiece dimer, the method comprising:

(a) transforming a host cell with a library of mutagenized DNA substrate molecules;

(b) inducing expression of the protein encoded by the library of (a);

(c) preparing an extract of the product of (b);

(d) fractionating insoluble protein from complexes of soluble protein and DNA; and

(e) recovering a DNA substrate molecule encoding an evolved protein from (d).

A further aspect of the invention is a method for evolving functional expression of a protein encoded by a DNA substrate molecule comprising a fusion of a DNA sequence encoding the protein with a DNA sequence encoding filamentous phage protein to generate a fusion protein, the method comprising:

(a) providing a host cell producing infectious particles expressing a fusion protein encoded by a library of mutagenized DNA substrate molecules;

(b) recovering from (a) infectious particles displaying the fusion protein;

(c) affinity purifying particles displaying the mutant protein using a ligand for the protein; and

(d) recovering a DNA substrate molecule encoding an evolved protein from affinity purified particles of (c).

A further aspect of the invention is a method for optimizing expression of a protein encoded by a DNA substrate molecule comprising a fusion of a DNA sequence encoding the protein with a lac headpiece dimer, wherein the DNA substrate molecule is present on a first plasmid vector, the method comprising:

(a) providing a host cell transformed with the first vector and a second vector comprising a library of mutants of at least one chaperonin gene, and at least one lac operator;

(b) preparing an extract of the product of (a);

(c) fractionating insoluble protein from complexes of soluble protein and DNA; and

(d) recovering DNA encoding a chaperonin gene from (c).

A further aspect of the invention is a method for optimizing expression of a protein encoded by a DNA substrate molecule comprising a fusion of a DNA sequence

encoding the protein with a filamentous phage gene, wherein the fusion is carried on a phagemid comprising a library of chaperonin gene mutants, the method comprising:

(a) providing a host cell producing infectious particles expressing a fusion protein encoded by a library of mutagenized DNA substrate molecules;

(b) recovering from (a) infectious particles displaying the fusion protein;

(c) affinity purifying particles displaying the protein using a ligand for the protein; and

(d) recovering DNA encoding the mutant chaperonin from affinity purified particles of (c).

A further aspect of the invention is a method for optimizing secretion of a protein in a host by evolving a gene encoding a secretory function, comprising:

(a) providing a cluster of genes encoding secretory functions;

(b) recombining at least a first and second sequence in the gene cluster of (a) encoding a secretory function, the at least a first and second sequences differing from each other in at least one nucleotide, to generate a library of recombinant sequences;

(c) transforming a host cell culture with the products of (b), wherein the host cell comprises a DNA sequence encoding the protein;

(d) subjecting the product of (c) to screening or selection for secretion of the protein; and

(e) recovering DNA encoding an evolved gene encoding a secretory function from the product of (d).

A further aspect of the invention is a method for evolving an improved DNA polymerase comprising:

(a) providing a library of mutant DNA substrate molecules encoding mutant DNA polymerase;

(b) screening extracts of cells transfected with (a) and comparing activity with wild type DNA polymerase;

(c) recovering mutant DNA substrate molecules from cells in (b) expressing mutant DNA polymerase having improved activity over wild-type DNA polymerase; and

(d) recovering a DNA substrate molecule encoding an evolved polymerase from the products of (c).

A further aspect of the invention is a method for evolving a DNA polymerase with an error rate greater than that of wild type DNA polymerase comprising:

(a) providing a library of mutant DNA substrate molecules encoding mutant DNA polymerase in a host cell comprising an indicator gene having a revertible mutation, wherein the indicator gene is replicated by the mutant DNA polymerase;

(b) screening the products of (a) for revertants of the indicator gene;

(c) recovering mutant DNA substrate molecules from revertants; and

(d) recovering a DNA substrate molecule encoding an evolved polymerase from the products of (c).

A further aspect of the invention is a method for evolving a DNA polymerase, comprising:

(a) providing a library of mutant DNA substrate molecules encoding mutant DNA polymerase, the library comprising a plasmid vector;

(b) preparing plasmid preparations and extracts of host cells transfected with the products of (a);

(c) amplifying each plasmid preparation in a PCR reaction using the mutant polymerase encoded by that plasmid, the polymerase being present in the host cell extract;

(d) recovering the PCR products of (c); and

(e) recovering a DNA substrate molecule encoding an evolved polymerase from the products of (d).

A further aspect of the invention is a method for evolving a p-nitrophenol phosphonatase from a phosphonatase encoded by a DNA substrate molecule, comprising:

(a) providing library of mutants of the DNA substrate molecule, the library comprising a plasmid expression vector;

(b) transfecting a host, wherein the host phn operon is deleted;

(c) selecting for growth of the transfectants of (b) using a p-nitrophenol phosphonatase as a substrate;

(d) recovering the DNA substrate molecules from transfectants selected from (c); and

(e) recovering a DNA substrate molecule from (d) encoding an evolved phosphonatase.

A further aspect of the invention is a method for evolving a protease encoded by a DNA substrate molecule comprising:

(a) providing library of mutants of the DNA substrate molecule, the library comprising a plasmid expression vector, wherein the DNA substrate molecule is linked to a secretory leader;

(b) transfecting a host;

(c) selecting for growth of the transfectants of (b) on a complex protein medium; and

(d) recovering a DNA substrate molecule from (c) encoding an evolved protease.

A further aspect of the invention is a method for screening a library of protease mutants displayed on a phage to obtain an improved protease, wherein a DNA

substrate molecule encoding the protease is fused to DNA encoding a filamentous phage protein to generate a fusion protein, comprising:

(a) providing host cells expressing the fusion protein;

(b) overlaying host cells with a protein net to entrap the phage;

(c) washing the product of (b) to recover phage liberated by digestion of the protein net;

(d) recovering DNA from the product of (c); and

(e) recovering a DNA substrate from (d) encoding an improved protease.

A further aspect of the invention is a method for screening a library of protease mutants to obtain an improved protease, the method comprising:

(a) providing a library of peptide substrates, the peptide substrate comprising a fluorophore and a fluorescence quencher;

(b) screening the library of protease mutants for ability to cleave the peptide substrates, wherein fluorescence is measured; and

(c) recovering DNA encoding at least one protease mutant from (b).

A further aspect of the invention is a method for evolving an alpha interferon gene comprising:

(a) providing a library of mutant alpha interferon genes, the library comprising a filamentous phage vector;

(b) stimulating cells comprising a reporter construct, the reporter construct comprising a reporter gene under control of an interferon responsive promoter, and wherein the reporter gene is GFP;

(c) separating the cells expressing GFP by FACS;

(d) recovering phage from the product of (c); and

(e) recovering an evolved interferon gene from the product of (d).

A further aspect of the invention is a method for screening a library of mutants of a DNA substrate encoding a protein for an evolved DNA substrate, comprising:

(a) providing a library of mutants, the library comprising an expression vector;

(b) transfecting a mammalian host cell with the library of (a), wherein mutant protein is expressed on the surface of the cell;

(c) screening or selecting the products of (b) with a ligand for the protein;

(d) recovering DNA encoding mutant protein from the products of (c); and

(e) recovering an evolved DNA substrate from the products of (d).

A further aspect of the invention is a method for evolving a DNA substrate molecule encoding an interferon alpha, comprising:

(a) providing a library of mutant alpha interferon genes, the library comprising an expression vector wherein the alpha interferon genes are expressed under the control of an inducible promoter;

(b) transfecting host cells with the library of (a);

(c) contacting the product of (b) with a virus;

(d) recovering DNA encoding a mutant alpha interferon from host cells surviving step (c); and

(e) recovering an evolved interferon gene from the product of (d).

A further aspect of the invention is a method for evolving the serum stability or circulation half-life of a protein encoded by a DNA substrate molecule, the DNA substrate molecule comprising a fusion of a DNA sequence encoding the protein with a DNA sequence encoding a filamentous phage protein to generate a fusion protein, the method comprising:

(a) providing a host cell expressing a library of mutants of the fusion protein;

(b) affinity purifying the mutants with a ligand for the protein, wherein the ligand is a human serum protein, tissue specific protein, or receptor;

(c) recovering DNA encoding a mutant protein from the affinity selected mutants of (b); and

(d) recovering an evolved gene encoding the protein from the product of (c).

A further aspect of the invention is a method for evolving a protein having at least two subunits, comprising:

(a) providing a library of mutant DNA substrate molecules for each subunit;

(b) recombining the libraries into a library of single chain constructs of the protein, the single chain construct comprising a DNA substrate molecule encoding each subunit sequence, the subunit sequence being linked by a linker at a nucleic acid sequence encoding the amino terminus of one subunit to a nucleic acid sequence encoding the carboxy terminus of a second subunit;

(c) screening or selecting the products of (B),

(d) recovering recombinant single chain construct DNA substrate molecules from the products of (c);

(e) subjecting the products of (d) to mutagenesis; and

(f) recovering an evolved single chain construct DNA substrate molecule from (e).

A further aspect of the invention is a method for evolving the coupling of a mammalian 7-transmembrane receptor to a yeast signal transduction pathway, comprising:

(a) expressing a library of mammalian G alpha protein mutants in a host cell, wherein the host cell expresses the mammalian 7-transmembrane receptor and a

reporter gene, the receptor gene geing expressed under control of a pheromone responsive promoter;

(b) screening or selecting the products of (a) for expression of the reporter gene in the presence of a ligand for the 7-transmembrance receptor; and

(c) recovering DNA encoding an evolved G alpha protein mutant from screened or selected products of (b).

A further aspect of the invention is a method for recombining at least a first and second DNA substrate molecule, comprising:

(a) transfecting a host cell with at least a first and second DNA substrate molecule wherein the at least a first and second DNA substrate molecules are recombined in the host cell;

(b) screening or selecting the products of (a) for a desired property; and

(c) recovering recombinant DNA substrate molecules from (b).

A further aspect of the invention is a method for evolving a DNA substrate sequence encoding a protein of interest, wherein the DNA substrate comprises a vector, the vector comprising single-stranded DNA, the method comprising:

(a) providing single-stranded vector DNA and a library of mutants of the DNA substrate sequence;

(b) annealing single stranded DNA from the library of (a) to the single stranded vector DNA of (a);

(c) transforming the products of (b) into a host;

(d) screening the product of (c) for a desired property; and

(e) recovering evolved DNA substrate DNA from the products of (d).

Brief Description of the Drawings

Figure 1 depicts the alignment of oligo PCR primers for evolution of bovine calf intestinal alkaline phosphatase.

Figure 2 depicts the alignment of alpha interferon amino acid and nucleic acid sequences.

Figure 3 depicts the alignment of chimeric alpha interferon amino acid sequences.

Description of the Specific Embodiments

The invention provides a number of strategies for evolving polypeptides through recursive recombination methods. In some embodiments, the strategies of the invention can generally be classified as "coarse grain shuffling" and "fine grain shuffling." As described in detail below, these strategies are especially applicable in situations where some structural or functional information is available regarding the polypeptides of interest, where

the nucleic acid to be manipulated is large, when selection or screening of many recombinants is cumbersome, and so on. "Coarse grain shuffling" generally involves the exchange or recombination of segments of nucleic acids, whether defined as functional domains, exons, restriction endonuclease fragments, or otherwise arbitrarily defined segments. "Fine grain shuffling" generally involves the introduction of sequence variation within a segment, such as within codons.

Coarse grain and fine grain shuffling allow analysis of variation occuring within a nucleic acid sequence, also termed "searching of sequence space." Although both techniques are meritorious, the results are qualitatively different. For example, coarse grain searches are often better suited for optimizing multigene clusters such as polyketide operons, whereas fine grain searches are often optimal for optimizing a property such as protein expression using codon usage libraries.

The strategies generally entail evolution of gene(s) or segment(s) thereof to allow retention of function in a heterologous cell or improvement of function in a homologous or heterologous cell. Evolution is effected generally by a process termed recursive sequence recombination. Recursive sequence recombination can be achieved in many different formats and permutations of formats, as described in further detail below. These formats share some common principles. Recursive sequence recombination entails successive cycles of recombination to generate molecular diversity, i.e., the creation of a family of nucleic acid molecules showing substantial sequence identity to each other but differing in the presence of mutations. Each recombination cycle is followed by at least one cycle of screening or selection for molecules having a desired characteristic. The molecule(s) selected in one round form the starting materials for generating diversity in the next round. In any given cycle, recombination can occur *in vivo* or *in vitro*. Furthermore, diversity resulting from recombination can be augmented in any cycle by applying prior methods of mutagenesis (e.g., error-prone PCR or cassette mutagenesis, passage through bacterial mutator strains, treatment with chemical mutagens, "spiking" with sequence diversity from homologous gene families) to either the substrates for or products of recombination.

I. **Formats for Recursive Sequence Recombination**

Some formats and examples for recursive sequence recombination, sometimes referred to as DNA shuffling, evolution, or molecular breeding, have been described by the present inventors and co-workers in co-pending applications U.S. Patent Application Serial No. 08/198,431, filed February 17, 1994, Serial No. PCT/US95/02126, filed, February 17, 1995, Serial No. 08/425,684, filed April 18, 1995, Serial No. 08/537,874, filed October 30, 1995, Serial No. 08/564,955, filed November 30, 1995. Serial No. 08/621,859, filed March 25, 1996, Serial No. 08/621,430, filed March 25, 1996, Serial No.

PCT/US96/05480, filed April 18, 1996, Serial No. 08/650,400, filed May 20, 1996, Serial No. 08/675,502, filed July 3, 1996, Serial No. 08/721, 824, filed September 27, 1996, and 08/722,660 filed September 27, 1996; Stemmer, Science 270:1510 (1995); Stemmer et al., Gene 164:49-53 (1995); Stemmer, Bio/Technology 13:549-553 (1995); Stemmer, Proc. Natl. Acad. Sci. U.S.A. 91:10747-10751 (1994); Stemmer, Nature 370:389-391 (1994); Crameri et al., Nature Medicine 2(1):1-3 (1996); Crameri et al., Nature Biotechnology 14:315-319 (1996), each of which is incorporated by reference in its entirety for all purposes.

In general, the term "gene" is used herein broadly to refer to any segment or sequence of DNA associated with a biological function. Genes can be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters.

A wide variety of cell types can be used as a recipient of evolved genes. Cells of particular interest include many bacterial cell types, both gram-negative and gram-positive, such as *Rhodococcus*, *Streptomycetes*, *Actinomycetes*, *Corynebacteria*, *Penicillium*, *Bacillus*, *Escherichia coli*, *Pseudomonas*, *Salmonella*, and *Erwinia*. Cells of interest also include eukaryotic cells, particularly mammalian cells (e.g., mouse, hamster, primate, human), both cell lines and primary cultures. Such cells include stem cells, including embryonic stem cells, zygotes, fibroblasts, lymphocytes, Chinese hamster ovary (CHO), mouse fibroblasts (NIH3T3), kidney, liver, muscle, and skin cells. Other eukaryotic cells of interest include plant cells, such as maize, rice, wheat, cotton, soybean, sugarcane, tobacco, and arabidopsis; fish, algae, fungi (*Penicillium*, *Fusarium*, *Aspergillus*, *Podospora*, *Neurospora*), insects, yeasts (*Picchia* and *Saccharomyces*).

The choice of host will depend on a number of factors, depending on the intended use of the engineered host, including pathogenicity, substrate range, environmental hardiness, presence of key intermediates, ease of genetic manipulation, and likelihood of promiscuous transfer of genetic information to other organisms. A preferred host has the ability to replicate vector DNA, express proteins of interest, and properly traffic proteins of interest. Particularly advantageous hosts are *E. coli*, *lactobacilli*, *Streptomycetes*, *Actinomycetes*, *fungi* such as *Saccaromyces cerivisiae* or *Pischia pastoris*, Schneider cells, L-cells, COS cells, CHO cells, and transformed B cell lines such as SP2/0, J558, NS-1 and AG8-653.

The breeding procedure starts with at least two substrates that generally show substantial sequence identity to each other (i.e., at least about 50%, 70%, 80% or 90% sequence identity), but differ from each other at certain positions. The difference can be any type of mutation, for example, substitutions, insertions and deletions. Often, different

segments differ from each other in perhaps 5-20 positions. For recombination to generate increased diversity relative to the starting materials, the starting materials must differ from each other in at least two nucleotide positions. That is, if there are only two substrates, there should be at least two divergent positions. If there are three substrates, for example, one substrate can differ from the second as a single position, and the second can differ from the third at a different single position. The starting DNA segments can be natural variants of each other, for example, allelic or species variants. The segments can also be from nonallelic genes showing some degree of structural and usually functional relatedness (e.g., different genes within a superfamily such as the immunoglobulin superfamily). The starting DNA segments can also be induced variants of each other. For example, one DNA segment can be produced by error-prone PCR replication of the other, or by substitution of a mutagenic cassette. Induced mutants can also be prepared by propagating one (or both) of the segments in a mutagenic strain. In these situations, strictly speaking, the second DNA segment is not a single segment but a large family of related segments. The different segments forming the starting materials are often the same length or substantially the same length. However, this need not be the case. For example; one segment can be a subsequence of another. The segments can be present as part of larger molecules, such as vectors, or can be in isolated form.

The starting DNA segments are recombined by any of the recursive sequence recombination formats provided herein to generate a diverse library of recombinant DNA segments. Such a library can vary widely in size from having fewer than 10 to more than 10^5, 10^9, or 10^{12} members. In general, the starting segments and the recombinant libraries generated include full-length coding sequences and any essential regulatory sequences, such as a promoter and polyadenylation sequence, required for expression. However, if this is not the case, the recombinant DNA segments in the library can be inserted into a common vector providing the missing sequences before performing screening/selection.

If the recursive sequence recombination format employed is an *in vivo* format, the library of recombinant DNA segments generated already exists in a cell, which is usually the cell type in which expression of the enzyme with altered substrate specificity is desired. If recursive sequence recombination is performed *in vitro*, the recombinant library is preferably introduced into the desired cell type before screening/selection. The members of the recombinant library can be linked to an episome or virus before introduction or can be introduced directly. In some embodiments of the invention, the library is amplified in a first host, and is then recovered from that host and introduced to a second host more amenable to expression, selection, or screening, or any other desirable parameter. The manner in which the library is introduced into the cell type depends on the DNA-uptake characteristics

of the cell type, e.g., having viral receptors, being capable of conjugation, or being naturally competent. If the cell type is insusceptible to natural and chemical-induced competence, but susceptible to electroporation, one would usually employ electroporation. If the cell type is insusceptible to electroporation as well, one can employ biolistics. The biolistic PDS-1000 Gene Gun (Biorad, Hercules, CA) uses helium pressure to accelerate DNA-coated gold or tungsten microcarriers toward target cells. The process is applicable to a wide range of tissues, including plants, bacteria, fungi, algae, intact animal tissues, tissue culture cells, and animal embryos. One can employ electronic pulse delivery, which is essentially a mild electroporation format for live tissues in animals and patients. Zhao, Advanced Drug Delivery Reviews 17:257-262 (1995). Novel methods for making cells competent are described in co-pending application U.S. Patent Application Serial No. 08/621,430, filed March 25, 1996. After introduction of the library of recombinant DNA genes, the cells are optionally propagated to allow expression of genes to occur.

A. ***In Vitro* Formats**

One format for recursive sequence recombination utilizes a pool of related sequences. The sequences can be DNA or RNA and can be of various lengths depending on the size of the gene or DNA fragment to be recombined or reassembled. Preferably the sequences are from 50 bp to 100 kb.

The pool of related substrates can be fragmented, usually at random, into fragments of from about 5 bp to 5 kb or more. Preferably the size of the random fragments is from about 10 bp to 1000 bp, more preferably the size of the DNA fragments is from about 20 bp to 500 bp. The substrates can be digested by a number of different methods, such as DNAseI or RNAse digestion, random shearing or restriction enzyme digestion. The concentration of nucleic acid fragments of a particular length is often less than 0.1 % or 1% by weight of the total nucleic acid. The number of different specific nucleic acid fragments in the mixture is usually at least about 100, 500 or 1000.

The mixed population of nucleic acid fragments are denatured by heating to about 80° C to 100° C, more preferably from 90° C to 96° C, to form single-stranded nucleic acid fragments. Single-stranded nucleic acid fragments having regions of sequence identity with other single-stranded nucleic acid fragments can then be reannealed by cooling to 6°C to 75°C, and preferably from 40°C to 65°C. Renaturation can be accelerated by the addition of polyethylene glycol ("PEG") or salt. The salt concentration is preferably from 0 mM to 600 mM, more preferably the salt concentration is from 10 mM to 100 mM. The salt may be such salts as $(NH_4)_2SO_4$, KCl, or NaCl. The concentration of PEG is preferably from 0% to 20%, more preferably from 5% to 10%. The fragments that reanneal can be from different substrates.

The annealed nucleic acid fragments are incubated in the presence of a nucleic acid polymerase, such as Taq or Klenow, Mg^{++} at 1mM - 20mM, and dNTP's (i.e. dATP, dCTP, dGTP and dTTP). If regions of sequence identity are large, Taq or other high-temperature polymerase can be used with an annealing temperature of between 45-65°C. If the areas of identity are small, Klenow or other polymerases that are active at low temperature can be used, with an annealing temperature of between 6-30°C. The polymerase can be added to the random nucleic acid fragments prior to annealing, simultaneously with annealing or after annealing.

The cycle of denaturation, renaturation and incubation of random nucleic acid fragments in the presence of polymerase is sometimes referred to as "shuffling" of the nucleic acid *in vitro*. This cycle is repeated for a desired number of times. Preferably the cycle is repeated from 2 to 100 times, more preferably the sequence is repeated from 10 to 40 times. The resulting nucleic acids are a family of double-stranded polynucleotides of from about 50 bp to about 100 kb, preferably from 500 bp to 50 kb. The population represents variants of the starting substrates showing substantial sequence identity thereto but also diverging at several positions. The population has many more members than the starting substrates. The population of fragments resulting from recombination is preferably first amplified by PCR, then cloned into an appropriate vector and the ligation mixture used to transform host cells.

In a variation of *in vitro* shuffling, subsequences of recombination substrates can be generated by amplifying the full-length sequences under conditions which produce a substantial fraction, typically at least 20 percent or more, of incompletely extended amplification products. The amplification products, including the incompletely extended amplification products are denatured and subjected to at least one additional cycle of reannealing and amplification. This variation, wherein at least one cycle of reannealing and amplification provides a substantial fraction of incompletely extended products, is termed "stuttering." In the subsequent amplification round, the incompletely extended products anneal to and prime extension on different sequence-related template species.

In a further variation, at least one cycle of amplification can be conducted using a collection of overlapping single-stranded DNA fragments of related sequence, and different lengths. Each fragment can hybridize to and prime polynucleotide chain extension of a second fragment from the collection, thus forming sequence-recombined polynucleotides. In a further variation, single-stranded DNA fragments of variable length can be generated from a single primer by Vent DNA polymerase on a first DNA template. The single stranded DNA fragments are used as primers for a second, Kunkel-type template, consisting of a uracil-containing circular single-stranded DNA. This results in multiple

substitutions of the first template into the second (see Levichkin et al., Mol. Biology 29:572-577 (1995)).

Nucleic acid sequences can be recombined by recursive sequence recombination even if they lack sequence homology. Homology can be introduced using synthetic oligonucleotides as PCR primers. In addition to the specific sequences for the nucleic acid segment being amplified, all of the primers used to amplify one particular segment are synthesized to contain an additional sequence of 20-40 bases 5' to the gene (sequence A) and a different 20-40 base sequence 3' to the segment (sequence B). An adjacent segment is amplified using a 5' primer which contains the complementary strand of sequence B (sequence B'), and a 3' primer containing a different 20-40 base sequence (C). Similarly, primers for the next adjacent segment contain sequences C' (complementary to C) and D. In this way, small regions of homology are introduced, making the segments into site-specific recombination cassettes. Subsequent to the initial amplification of individual segments, the amplified segments can then be mixed and subjected to primerless PCR.

When domains within a polypeptide are shuffled, it may not be possible to introduce additional flanking sequences to the domains, due to the constraint of maintaining a continuous open reading frame. Instead, groups of oligonucleotides are synthesized that are homologous to the 3' end of the first domain encoded by one of the genes to be shuffled, and the 5' ends of the second domains encoded by all of the other genes to be shuffled together. This is repeated with all domains, thus providing sequences that allow recombination between protein domains while maintaining their order.

B. *In Vivo* Formats

1. Plasmid-Plasmid Recombination

The initial substrates for recombination are a collection of polynucleotides comprising variant forms of a gene. The variant forms usually show substantial sequence identity to each other sufficient to allow homologous recombination between substrates. The diversity between the polynucleotides can be natural (e.g., allelic or species variants), induced (e.g., error-prone PCR or error-prone recursive sequence recombination), or the result of *in vitro* recombination. Diversity can also result from resynthesizing genes encoding natural proteins with alternative codon usage. There should be at least sufficient diversity between substrates that recombination can generate more diverse products than there are starting materials. There must be at least two substrates differing in at least two positions. However, commonly a library of substrates of 10^3-10^8 members is employed. The degree of diversity depends on the length of the substrate being recombined and the extent of the functional change to be evolved. Diversity at between 0.1-25% of positions is typical. The diverse substrates are incorporated into plasmids. The plasmids are often standard cloning

vectors, e.g., bacterial multicopy plasmids. However, in some methods to be described below, the plasmids include mobilization (MOB) functions. The substrates can be incorporated into the same or different plasmids. Often at least two different types of plasmid having different types of selectable markers are used to allow selection for cells containing at least two types of vector. Also, where different types of plasmid are employed, the different plasmids can come from two distinct incompatibility groups to allow stable co-existence of two different plasmids within the cell. Nevertheless, plasmids from the same incompatibility group can still co-exist within the same cell for sufficient time to allow homologous recombination to occur.

Plasmids containing diverse substrates are initially introduced into cells by any method (e.g., chemical transformation, natural competence, electroporation, biolistics, packaging into phage or viral systems). Often, the plasmids are present at or near saturating concentration (with respect to maximum transfection capacity) to increase the probability of more than one plasmid entering the same cell. The plasmids containing the various substrates can be transfected simultaneously or in multiple rounds. For example, in the latter approach cells can be transfected with a first aliquot of plasmid, transfectants selected and propagated, and then infected with a second aliquot of plasmid.

Having introduced the plasmids into cells, recombination between substrates to generate recombinant genes occurs within cells containing multiple different plasmids merely by propagating the cells. However, cells that receive only one plasmid are unable to participate in recombination and the potential contribution of substrates on such plasmids to evolution is not fully exploited (although these plasmids may contribute to some extent if they are progagated in mutator cells). The rate of evolution can be increased by allowing all substrates to participate in recombination. Such can be achieved by subjecting transfected cells to electroporation. The conditions for electroporation are the same as those conventionally used for introducing exogenous DNA into cells (e.g., 1,000-2,500 volts, 400 μF and a 1-2 mM gap). Under these conditions, plasmids are exchanged between cells allowing all substrates to participate in recombination. In addition the products of recombination can undergo further rounds of recombination with each other or with the original substrate. The rate of evolution can also be increased by use of conjugative transfer. To exploit conjugative transfer, substrates can be cloned into plasmids having MOB genes, and *tra* genes are also provided in *cis* or in *trans* to the MOB genes. The effect of conjugative transfer is very similar to electroporation in that it allows plasmids to move between cells and allows recombination between any substrate and the products of previous recombination to occur, merely by propagating the culture. The rate of evolution can also be increased by fusing cells to induce exchange of plasmids or chromosomes. Fusion can be

induced by chemical agents, such as PEG, or viral proteins, such as influenza virus hemagglutinin, HSV-1 gB and gD. The rate of evolution can also be increased by use of mutator host cells (e.g., Mut L, S, D, T, H in bacteria and Ataxia telangiectasia human cell lines).

The time for which cells are propagated and recombination is allowed to occur, of course, varies with the cell type but is generally not critical, because even a small degree of recombination can substantially increase diversity relative to the starting materials. Cells bearing plasmids containing recombined genes are subject to screening or selection for a desired function. For example, if the substrate being evolved contains a drug resistance gene, one would select for drug resistance. Cells surviving screening or selection can be subjected to one or more rounds of screening/selection followed by recombination or can be subjected directly to an additional round of recombination. "Screening" as used herein is intended to include "selection" as a type of screen.

The next round of recombination can be achieved by several different formats independently of the previous round. For example, a further round of recombination can be effected simply by resuming the electroporation or conjugation-mediated intercellular transfer of plasmids described above. Alternatively, a fresh substrate or substrates, the same or different from previous substrates, can be transfected into cells surviving selection/screening. Optionally, the new substrates are included in plasmid vectors bearing a different selective marker and/or from a different incompatibility group than the original plasmids. As a further alternative, cells surviving selection/screening can be subdivided into two subpopulations, and plasmid DNA from one subpopulation transfected into the other, where the substrates from the plasmids from the two subpopulations undergo a further round of recombination. In either of the latter two options, the rate of evolution can be increased by employing DNA extraction, electroporation, conjugation or mutator cells, as described above. In a still further variation, DNA from cells surviving screening/selection can be extracted and subjected to *in vitro* recursive sequence recombination.

After the second round of recombination, a second round of screening/selection is performed, preferably under conditions of increased stringency. If desired, further rounds of recombination and selection/screening can be performed using the same strategy as for the second round. With successive rounds of recombination and selection/ screening, the surviving recombined substrates evolve toward acquisition of a desired phenotype. Typically, in this and other methods of recursive recombination, the final product of recombination that has acquired the desired phenotype differs from starting substrates at 0.1%-25% of positions and has evolved at a rate orders of magnitude in excess (e.g., by at least 10-fold, 100-fold, 1000-fold, or 10,000 fold) of the rate of evolution driven by

naturally acquired mutation of about 1 mutation per 10^5 positions per generation (see Anderson et al., Proc. Natl. Acad. Sci. U.S.A. 93:906-907 (1996)). The "final product" may be transferred to another host more desirable for utilization of the "shuffled" DNA. This is particularly advantageous in situations where the more desirable host is less efficient as a host for the many cycles of mutation/ recombination due to the lack of molecular biology or genetic tools available for other organisms such as *E. coli*.

2. Virus-Plasmid Recombination

The strategy used for plasmid-plasmid recombination can also be used for virus-plasmid recombination; usually, phage-plasmid recombination. However, some additional comments particular to the use of viruses are appropriate. The initial substrates for recombination are cloned into both plasmid and viral vectors. It is usually not critical which substrate(s) is/are inserted into the viral vector and which into the plasmid, although usually the viral vector should contain different substrate(s) from the plasmid. As before, the plasmid (and the virus) typically contains a selective marker. The plasmid and viral vectors can both be introduced into cells by transfection as described above. However, a more efficient procedure is to transfect the cells with plasmid, select transfectants and infect the transfectants with virus. Because the efficiency of infection of many viruses approaches 100% of cells, most cells transfected and infected by this route contain both a plasmid and virus bearing different substrates.

Homologous recombination occurs between plasmid and virus generating both recombined plasmids and recombined virus. For some viruses, such as filamentous phage, in which intracellular DNA exists in both double-stranded and single-stranded forms, both can participate in recombination. Provided that the virus is not one that rapidly kills cells, recombination can be augmented by use of electroporation or conjugation to transfer plasmids between cells. Recombination can also be augmented for some types of virus by allowing the progeny virus from one cell to reinfect other cells. For some types of virus, virus infected-cells show resistance to superinfection. However, such resistance can be overcome by infecting at high multiplicity and/or using mutant strains of the virus in which resistance to superinfection is reduced.

The result of infecting plasmid-containing cells with virus depends on the nature of the virus. Some viruses, such as filamentous phage, stably exist with a plasmid in the cell and also extrude progeny phage from the cell. Other viruses, such as lambda having a cosmid genome, can stably exist in a cell like plasmids without producing progeny virions. Other viruses, such as the T-phage and lytic lambda, undergo recombination with the plasmid but ultimately kill the host cell and destroy plasmid DNA. For viruses that infect cells without killing the host, cells containing recombinant plasmids and virus can be

screened/selected using the same approach as for plasmid-plasmid recombination. Progeny virus extruded by cells surviving selection/screening can also be collected and used as substrates in subsequent rounds of recombination. For viruses that kill their host cells, recombinant genes resulting from recombination reside only in the progeny virus. If the screening or selective assay requires expression of recombinant genes in a cell, the recombinant genes should be transferred from the progeny virus to another vector, e.g., a plasmid vector, and retransfected into cells before selection/screening is performed.

For filamentous phage, the products of recombination are present in both cells surviving recombination and in phage extruded from these cells. The dual source of recombinant products provides some additional options relative to the plasmid-plasmid recombination. For example, DNA can be isolated from phage particles for use in a round of *in vitro* recombination. Alternatively, the progeny phage can be used to transfect or infect cells surviving a previous round of screening/selection, or fresh cells transfected with fresh substrates for recombination.

3. Virus-Virus Recombination

The principles described for plasmid-plasmid and plasmid-viral recombination can be applied to virus-virus recombination with a few modifications. The initial substrates for recombination are cloned into a viral vector. Usually, the same vector is used for all substrates. Preferably, the virus is one that, naturally or as a result of mutation, does not kill cells. After insertion, some viral genomes can be packaged *in vitro* or using a packaging cell line. The packaged viruses are used to infect cells at high multiplicity such that there is a high probability that a cell will receive multiple viruses bearing different substrates.

After the initial round of infection, subsequent steps depend on the nature of infection as discussed in the previous section. For example, if the viruses have phagemid (Sambrook et al., Molecular Cloning, CSH Press, 1987) genomes such as lambda cosmids or M13, F1 or Fd phagemids, the phagemids behave as plasmids within the cell and undergo recombination simply by propagating within the cells. Recombination is particularly efficient between single-stranded forms of intracellular DNA. Recombination can be augmented by electroporation of cells.

Following selection/screening, cosmids containing recombinant genes can be recovered from surviving cells, e.g., by heat induction of a cos$^-$ lysogenic host cell, or extraction of DNA by standard procedures, followed by repackaging cosmid DNA *in vitro*.

If the viruses are filamentous phage, recombination of replicating form DNA occurs by propagating the culture of infected cells. Selection/screening identifies colonies of cells containing viral vectors having recombinant genes with improved properties, together

with infectious particles (i.e., phage or packaged phagemids) extruded from such cells. Subsequent options are essentially the same as for plasmid-viral recombination.

4. Chromosome Recombination

This format can be used to especially evolve chromosomal substrates. The format is particularly preferred in situations in which many chromosomal genes contribute to a phenotype or one does not know the exact location of the chromosomal gene(s) to be evolved. The initial substrates for recombination are cloned into a plasmid vector. If the chromosomal gene(s) to be evolved are known, the substrates constitute a family of sequences showing a high degree of sequence identity but some divergence from the chromosomal gene. If the chromosomal genes to be evolved have not been located, the initial substrates usually constitute a library of DNA segments of which only a small number show sequence identity to the gene or gene(s) to be evolved. Divergence between plasmid-borne substrate and the chromosomal gene(s) can be induced by mutagenesis or by obtaining the plasmid-borne substrates from a different species than that of the cells bearing the chromosome.

The plasmids bearing substrates for recombination are transfected into cells having chromosomal gene(s) to be evolved. Evolution can occur simply by propagating the culture, and can be accelerated by transferring plasmids between cells by conjugation or electroporation. Evolution can be further accelerated by use of mutator host cells or by seeding a culture of nonmutator host cells being evolved with mutator host cells and inducing intercellular transfer of plasmids by electroporation or conjugation. Preferably, mutator host cells used for seeding contain a negative selectable marker to facilitate isolation of a pure culture of the nonmutator cells being evolved. Selection/screening identifies cells bearing chromosomes and/or plasmids that have evolved toward acquisition of a desired function.

Subsequent rounds of recombination and selection/screening proceed in similar fashion to those described for plasmid-plasmid recombination. For example, further recombination can be effected by propagating cells surviving recombination in combination with electroporation or conjugative transfer of plasmids. Alternatively, plasmids bearing additional substrates for recombination can be introduced into the surviving cells. Preferably, such plasmids are from a different incompatibility group and bear a different selective marker than the original plasmids to allow selection for cells containing at least two different plasmids. As a further alternative, plasmid and/or chromosomal DNA can be isolated from a subpopulation of surviving cells and transfected into a second subpopulation. Chromosomal DNA can be cloned into a plasmid vector before transfection.

5. Virus-Chromosome Recombination

As in the other methods described above, the virus is usually one that does not kill the cells, and is often a phage or phagemid. The procedure is substantially the same as for plasmid-chromosome recombination. Substrates for recombination are cloned into the vector. Vectors including the substrates can then be transfected into cells or *in vitro* packaged and introduced into cells by infection. Viral genomes recombine with host chromosomes merely by propagating a culture. Evolution can be accelerated by allowing intercellular transfer of viral genomes by electroporation, or reinfection of cells by progeny virions. Screening/selection identifies cells having chromosomes and/or viral genomes that have evolved toward acquisition of a desired function.

There are several options for subsequent rounds of recombination. For example, viral genomes can be transferred between cells surviving selection/recombination by electroporation. Alternatively, viruses extruded from cells surviving selection/screening can be pooled and used to superinfect the cells at high multiplicity. Alternatively, fresh substrates for recombination can be introduced into the cells, either on plasmid or viral vectors.

II. Application of Recursive Sequence Recombination to Evolution of Polypeptides

In addition to the techniques described above, some additionally advantageous modifications of these techniques for the evolution of polypeptides are described below. These methods are referred to as "fine grain" and "coarse grain" shuffling. The coarse grain methods allow one to exchange chunks of genetic material between substrate nucleic acids, thereby limiting diversity in the resulting recombinants to exchanges or substitutions of domains, restriction fragments, oligo-encoded blocks of mutations, or other arbitrarily defined segments, rather than introducing diversity more randomly across the substrate. In contrast to coarse grain shuffling, fine grain shuffling methods allow the generation of all possible recombinations, or permutations, of a given set of very closely linked mutations, including multiple permutations, within a single segment, such as a codon.

In some embodiments, coarse grain or fine grain shuffling techniques are not performed as exhaustive searches of all possible mutations within a nucleic acid sequence. Rather, these techniques are utilized to provide a sampling of variation possible within a gene based on known sequence or structural information. The size of the sample is typically determined by the nature of the screen or selection process. For example, when a screen is performed in a 96-well microtiter format, it may be preferable to limit the size of the recombinant library to about 100 such microtiter plates for convenience in screening.

The techniques described herein are especially useful in the recombination of genes from gene families, wherein diversity in nucleotide sequence is provided all or in part by naturally occurring differences in the nucleotide sequence of the genes in the family.

A "gene family" as used herein is intended to include genes with similar function, such as but not limited to interferons or interleukins; genes which are believed to be derived by descent from a common ancestor; and genes which encode proteins that are structurally homologous, such as four helix bundle proteins.

Thus, for example, DNA or protein sequences can be aligned by computer algorithms, such as those described in the monograph on bioinformatics by Schomburg and Lessel (Schomburg and Lessel, Bioinformatics: From Nucleic Acids and Proteins to Cell Metabolism, October 9 - 11, 1995, Braunschweig, Germany). These algorithms can determine the likelihood that two sequences, or subdomains of sequences, are related to each other by descent from a common ancestor. Sequences that are judged to be derived by descent from a common ancestor comprise a "homologous gene family", and DNA shuffling can be used to accelerate the evolution of these gene families.

Furthermore, many distinct protein sequences are consistent with similar protein folds, and such families of sequences can be said to comprise "structurally homologous" gene families. The superfamily of four helix bundle proteins are such a family. Although this is a very large family of functionally highly diverse proteins ranging from cytokines to enzymes to DNA binding proteins having this fold, it is unlikely that these proteins are derived from a common ancestor. It is more likely that they have "convergently evolved" to have similar protein folds. There are now functional algorithms (Dahiyat et al., Science 278:82-87 (1997)) that allow one to design proteins with desired protein folds, and such algorithms have been used to design, for example, zinc finger motifs that are not related in primary sequence to any known natural proteins.

A. **Use of Restriction Enzyme Sites to Recombine Mutations**

In some situations it is advantageous to use restriction enzyme sites in nucleic acids to direct the recombination of mutations in a nucleic acid sequence of interest. These techniques are particularly preferred in the evolution of fragments that cannot readily be shuffled by existing methods due to the presence of repeated DNA or other problematic primary sequence motifs. They are also preferred for shuffling large fragments (typically greater than 10 kb), such as gene clusters that cannot be readily shuffled and "PCR-amplified" because of their size. Although fragments up to 50 kb have been reported to be amplified by PCR (Barnes, Proc. Natl. Acad. Sci. (U.S.A.) 91:2216-2220 (1994)), it can be problematic for fragments over 10 kb, and thus alternative methods for shuffling in the range of 10 - 50 kb and beyond are preferred. Preferably, the restriction endonucleases used are

of the Class II type (Sambrook et al., Molecular Cloning, CSH Press, 1987) and of these, preferably those which generate nonpalindromic sticky end overhangs such as Alwn I, Sfi I or BstX1. These enzymes generate nonpalindromic ends that allow for efficient ordered reassembly with DNA ligase. Typically, restriction enzyme (or endonuclease) sites are identified by conventional restriction enzyme mapping techniques (Sambrook et al., Molecular Cloning, CSH Press, 1987), by analysis of sequence information for that gene, or by introduction of desired restriction sites into a nucleic acid sequence by synthesis (i.e. by incorporation of silent mutations).

The DNA substrate molecules to be digested can either be from *in vivo* replicated DNA, such as a plasmid preparation, or from PCR amplified nucleic acid fragments harboring the restriction enzyme recognition sites of interest, preferably near the ends of the fragment. Typically, at least two variants of a gene of interest, each having one or more mutations, are digested with at least one restriction enzyme determined to cut within the nucleic acid sequence of interest. The restriction fragments are then joined with DNA ligase to generate full length genes having shuffled regions. The number of regions shuffled will depend on the number of cuts within the nucleic acid sequence of interest. The shuffled molecules can be introduced into cells as described above and screened or selected for a desired property. Nucleic acid can then be isolated from pools (libraries) or clones having desired properties and subjected to the same procedure until a desired degree of improvement is obtained.

In some embodiments, at least one DNA substrate molecule or fragment thereof is isolated and subjected to mutagenesis. In some embodiments, the pool or library of religated restriction fragments are subjected to mutagenesis before the digestion-ligation process is repeated. "Mutagenesis" as used herein comprises such techniques known in the art as PCR mutagenesis, oligonucleotide-directed mutagenesis, site-directed mutagenesis, etc., and recursive sequence recombination by any of the techniques described herein.

An example of the use of this format is in the manipulation of polyketide clusters. Polyketide clusters (Khosla et al., TIBTECH 14, September 1996) are typically 10 to 100 kb in length, specifying multiple large polypeptides which assemble into very large multienzyme complexes. Due to the modular nature of these complexes and the modular nature of the biosynthetic pathway, nucleic acids encoding protein modules can be exchanged between different polyketide clusters to generate novel and functional chimeric polyketides. The introduction of rare restriction endonuclease sites such as SfiI (eight base recognition, nonpalindromic overhangs) at nonessential sites between polypeptides or in introns engineered within polypeptides would provide "handles" with which to manipulate exchange of nucleic acid segments using the technique described above.

B. Reassembly PCR

A further technique for recursively recombining mutations in a nucleic acid sequence utilizes "reassembly PCR". This method can be used to assemble multiple segments that have been separately evolved into a full length nucleic acid template such as a gene. This technique is performed when a pool of advantageous mutants is known from previous work or has been identified by screening mutants that may have been created by any mutagenesis technique known in the art, such as PCR mutagenesis, cassette mutagenesis, doped oligo mutagenesis, chemical mutagenesis, or propagation of the DNA template in vivo in mutator strains. Boundaries defining segments of a nucleic acid sequence of interest preferably lie in intergenic regions, introns, or areas of a gene not likely to have mutations of interest. Preferably, oligonucleotide primers (oligos) are synthesized for PCR amplification of segments of the nucleic acid sequence of interest, such that the sequences of the oligonucleotides overlap the junctions of two segments. The overlap region is typically about 10 to 100 nucleotides in length. Each of the segments is amplified with a set of such primers. The PCR products are then "reassembled" according to assembly protocols such as those used in Sections IA-B above to assemble randomly fragmented genes. In brief, in an assembly protocol the PCR products are first purified away from the primers, by, for example, gel electrophoresis or size exclusion chromatography. Purified products are mixed together and subjected to about 1-10 cycles of denaturing, reannealing, and extension in the presence of polymerase and deoxynucleoside triphosphates (dNTP's) and appropriate buffer salts in the absence of additional primers ("self-priming"). Subsequent PCR with primers flanking the gene are used to amplify the yield of the fully reassembled and shuffled genes. This method is necessarily "coarse grain" and hence only recombines mutations in a blockwise fashion, an advantage for some searches such as when recombining allelic variants of multiple genes within an operon.

In some embodiments, the resulting reassembled genes are subjected to mutagenesis before the process is repeated.

In some embodiments, oligonucleotides that incorporate uracil into the primers are used for PCR amplification. Typically uracil is incorporated at one site in the oligonucleotide. The products are treated with uracil glycosylase, thereby generating a single-stranded overhang, and are reassembled in an ordered fashion by a method such as disclosed by Rashtchian (Current Biology, 6:30-36 (1995)).

In a further embodiment, the PCR primers for amplification of segments of the nucleic acid sequence of interest are used to introduce variation into the gene of interest as follows. Mutations at sites of interest in a nucleic acid sequence are identified by screening or selection, by sequencing homologues of the nucleic acid sequence, and so on.

Oligonucleotide PCR primers are then synthesized which encode wild type or mutant information at sites of interest. These primers are then used in PCR mutagenesis to generate libraries of full length genes encoding permutations of wild type and mutant information at the designated positions. This technique is typically advantagous in cases where the screening or selection process is expensive, cumbersome, or impractical relative to the cost of sequencing the genes of mutants of interest and synthesizing mutagenic oligonucleotides.

An example of this method is the evolution of an improved Taq polymerase, as described in detail below. Mutant proteins resulting from application of the method are identified and assayed in a sequencing reaction to identify mutants with improved sequencing properties. This is typically done in a high throughput format (see, for example, Broach et al. Nature 384 (Supp): 14-16 (1996)) to yield, after screening, a small number, e.g., about 2 to 100, of candidate recombinants for further evaluation. The mutant genes can then be sequenced to provide information regarding the location of the mutation. The corresponding mutagenic oligonucleotide primers can be synthesized from this information, and used in a reassembly reaction as described above to efficiently generate a library with an average of many mutations per gene. One or more rounds of this protocol allows the efficient search for improved variants of the Taq polymerase.

C. **Enrichment for Mutant Sequence Information**

In some embodiments of the invention, recombination reactions, such as those discussed above, are enriched for mutant sequences so that the multiple mutant spectrum, i.e. possible combinations of mutations, is more efficiently sampled. The rationale for this is as follows. Assume that a number, n, of mutant clones with improved activity is obtained, wherein each clone has a single point mutation at a different position in the nucleic acid sequence. If this population of mutant clones with an average of one mutation of interest per nucleic acid sequence is then put into a recombination reaction, the resulting population will still have an average of one mutation of interest per nucleic acid sequence as defined by a Poisson distribution, leaving the multiple mutation spectrum relatively unpopulated.

The amount of screening required to identify recombinants having two or more mutations can be dramatically reduced by the following technique. The nucleic acid sequences of interest are obtained from a pool of mutant clones and prepared as fragments, typically by digestion with a restriction endonuclease , sonication, or by PCR amplification. The fragments are denatured, then allowed to reanneal, thereby generating mismatched hybrids where one strand of a mutant has hybridized with a complementary strand from a different mutant or wild-type clone. The reannealed products are then fragmented into

fragments of about 20 - 100 bp. for example, by the use of DNAseI. This fragmentation reaction has the effect of segregating regions of the template containing mismatches (mutant information) from those encoding wild type sequence. The mismatched hybrids can then be affinity purified using aptamers, dyes, or other agents which bind to mismatched DNA. A preferred embodiment is the use of mutS protein affinity matrix (Wagner et al., Nucleic Acids Res. 23(19):3944-3948 (1995); Su et al., Proc. Natl. Acad. Sci. (U.S.A.), 83:5057-5061(1986)) with a preferred step of amplifying the affinity-purified material in vitro prior to an assembly reaction. This amplified material is then put into a assembly PCR reaction as decribed above. Optionally, this material can be titrated against the original mutant pool (e.g., from about 100% to 10% of the mutS enriched pool) to control the average number of mutations per clone in the next round of recombination.

Another application of this method is in the assembly of gene constructs that are enriched for polymorphic bases occurring as natural or selected allelic variants or as differences between homologous genes of related species. For example, one may have several varieties of a plant that are believed to have heritable variation in a trait of interest (e.g., drought resistance). It then is of interest to construct a library of these variant genes containing many mutations per gene. MutS selection can be applied in combination with the assembly techniques described herein to generate such a pool of recombinants that are highly enriched for polymorphic ("mutant") information. In some embodiments, the pool of recombinant genes is provided in a transgenic host. Recombinants can be further evolved by PCR amplification of the transgene from transgenic organisms that are determined to have an improved phenotype and applying the formats described in this invention to further evolve them.

D. Intron-driven Recombination

In some instances, the substrate molecules for recombination have uniformly low homology, sporadically distributed regions of homology, or the region of homology is relatively small (for example, about 10 - 100 bp), such as phage displayed peptide ligands. These factors can reduce the efficiency and randomness of recombination in RSR. In some embodiments of the invention, this problem is addressed by the introduction of introns between coding exons in sequences encoding protein homologues. In further embodiments of the invention, introns can be used (Chong et al., J. Biol. Chem., 271:22159-22168 (1996)).

In this method, a nucleic acid sequence, such as a gene or gene family, is arbitrarily defined to have segments. The segments are preferably exons. Introns are engineered between the segments. Preferably, the intron inserted between the first and second segments is at least about 10% divergent from the intron inserted between second and third segments, the intron inserted between second and third segments is at least about

10% divergent from the introns inserted between any of the previous segment pairs, and so on through segments n and n+1. The introns between any given set of exons will thus initially be identical between all clones in the library, whereas the exons can be arbitrarily divergent in sequence. The introns therefore provide homologous DNA sequences that will permit application of any of the described methods for RSR while the exons can be arbitrarily small or divergent in sequence, and can evolve to achieve an arbitrarily large degree of sequence divergence without a significant loss in efficiency in recombination. Restriction sites can also be engineered into the intronic nucleic acid sequence of interest so as to allow a directed reassemmbly of restriction fragments. The starting exon DNA may be synthesized de novo from sequence information, or may be present in any nucleic acid preparation (e.g., genomic, cDNA, libraries, and so on). For example, 1 to 10 nonhomologous introns can be designed to direct recombination of the nucleic acid sequences of interest by placing them between exons. The sequence of the introns can be all or partly obtained from known intron sequence. Preferably, the introns are self-splicing. Ordered sets of introns and exon libraries are assembled into functional genes by standard methods (Sambrook et al., Molecular Cloning, CSH Press (1987)).

Any of the formats for in vitro or in vivo recombination described herein can be applied for recursive exon shuffling. A preferred format is to use nonpalindromic restriction sites such as Sfi I placed into the intronic sequences to promote shuffling. Pools of selected clones are digested with Sfi I and religated. The nonpalindromic overhangs promote ordered reassembly of the shuffled exons. These libraries of genes can be expressed and screened for desired properties, then subjected to further recursive rounds of recombination by this process. In some embodiments, the libraries are subjected to mutagenesis before the process is repeated.

An example of how the introduction of an intron into a mammalian library format would be used advantageously is as follows. An intron containing a lox (Sauer et al., Proc. Natl. Acad. Sci. (U.S.A.), 85:5166-5170 (1988)) site is arbitrarily introduced between amino acids 92 and 93 in each alpha interferon parental substrate. A library of 10^4 chimeric interferon genes is made for each of the two exons (residues 1-92 and residues 93-167), cloned into a replicating plasmid vector, and introduced into target cells. The number 10^4 is arbitrarily chosen for convenience in screening. An exemplary vector for expression in mammalian cells would contain an SV40 origin, with the host cells expressing SV40 large T antigen, so as to allow transient expression of the interferon constructs. The cells are challenged with a cytopathic virus such as vesicular stomatitis virus (VSV) in an interferon protection assay (e.g., Meister et al., J. Gen. Virol. 67:1633-1643, (1986)). Cells surviving due to expression of interferon are recovered, the two libraries of interferon genes are PCR

amplified, and recloned into a vector that can be amplified in *E. coli*. The amplified plasmids are then transfected at high multiplicity (e.g. 10 micrograms of plasmid per 10^6 cells) into a cre expressing host that can support replication of that vector. The presence of cre in the host cells promotes efficient recombination at the lox site in the interferon intron, thus shuffling the selected sets of exons. This population of cells is then used in a second round of selection by viral challenge and the process is applied recursively. In this format, the cre recombinase is preferrably expressed transiently on a cotransfected molecule that cannot replicate in the host. Thus, after segregation of recombinants from the cre expressing plasmid, no further recombination will occur and selection can be performed on genetically stable exon permutations. The method can be used with more than one intron, with recombination enhancing sequences other than cre/lox (e.g., int/xis, etc.), and with other vector systems such as but not limited to retroviruses, adenovirus or adeno- associated virus.

5. Synthetic Oligonucleotide Mediated Recombination

1. Oligo bridge across sequence space

In some embodiments of the invention, a search of a region of sequence space defined by a set of substrates, such as members of a gene family, having less than about 80%, more typically, less than about 50% homology, is desired. This region, which can be part or all of a gene or a gene is arbitrarily delineated into segments. The segment borders can be chosen randomly, based on correspondence with natural exons, based on structural considerations (loops, alpha helices, subdomains, whole domains, hydrophobic core, surface, dynamic simulations), and based on correlations with genetic mapping data.

Typically, the segments are then amplified by PCR with a pool of "bridge" oligonucleotides at each junction. Thus, if the set of five genes is broken into three segments A, B and C, and if there are five versions of each segment (A1, A2, ... C4, C5), twenty five oligonucleotides are made for each strand of the A-B junctions where each bridge oligo has 20 bases of homology to one of the A and one of the B segments. In some cases, the number of required oligonucleotides can be reduced by choosing segment boundaries that are identical in some or all of the gene family members. Oligonucleotides are similarly synthesized for the B-C junction. The family of A domains is amplified by PCR with an outside generic A primer and the pool of A-B junction oligonucleotides; the B domains with the A-B plus the B-C bridge oligonucleotides, and the C domains with the B-C bridge oligonucleotides plus a generic outside primer. Full length genes are made then made by assembly PCR or by the dUTP/uracil glycosylase methods described above. Preferably, products from this step are subjected to mutagenesis before the process of selection and recombination is repeated, until a desired level of improvement or the evolution of a desired

property is obtained. This is typically determined using a screening or selection as appropriate for the protein and property of interest.

An illustration of this method is illustrated below for the recombination of eleven homologous human alpha interferon genes.

2. Site Directed Mutagenesis (SDM) with Oligonucleotides Encoding Homologue Mutations Followed by Shuffling

In some embodiments of the invention, sequence information from one or more substrate sequences is added to a given "parental" sequence of interest, with subsequent recombination between rounds of screening or selection. Typically, this is done with site-directed mutagenesis performed by techniques well known in the art (Sambrook et al., Molecular Cloning, CSH Press (1987)) with one substrate as template and oligonucleotides encoding single or multiple mutations from other substrate sequences, e.g. homologous genes. After screening or selection for an improved phenotype of interest, the selected recombinant(s) can be further evolved using RSR techniques described herein. After screening or selection, site-directed mutagenesis can be done again with another collection of oligonucleotides encoding homologue mutations, and the above process repeated until the desired properties are obtained.

When the difference between two homologues is one or more single point mutations in a codon, degenerate oligonucleotides can be used that encode the sequences in both homologues. One oligo may include many such degenerate codons and still allow one to exhaustively search all permutations over that block of sequence. An example of this is provided below for the evolution of alpha interferon genes.

When the homologue sequence space is very large, it can be advantageous to restrict the search to certain variants. Thus, for example, computer modelling tools (Lathrop et al., J. Mol. Biol., 255:641-665 (1996)) can be used to model each homologue mutation onto the target protein and discard any mutations that are predicted to grossly disrupt structure and function.

F. Recombination Directed by Host Machinery

In some embodiments of the invention, DNA substrate molecules are introduced into cells, wherein the cellular machinery directs their recombination. For example, a library of mutants is constructed and screened or selected for mutants with improved phenotypes by any of the techniques described herein. The DNA substrate molecules encoding the best candidates are recovered by any of the techniques described herein, then fragmented and used to transfect a mammalian host and screened or selected for improved function. The DNA substrate molecules are recovered from the mammalian host, such as by PCR, and the process is repeated until a desired level of improvement is

obtained. In some embodiments, the fragments are denatured and reannealed prior to transfection, coated with recombination stimulating proteins such as recA, or co-transfected with a selectable marker such as Neo^R to allow the positive selection for cells receiving recombined versions of the gene of interest.

For example, this format is preferred for the in vivo affinity maturation of an antibody by RSR. In brief, a library of mutant antibodies is generated, as described herein for the 48G7 affinity maturation. This library is FACS purified with ligand to enrich for antibodies with the highest 0.1 - 10% affinity. The V regions genes are recovered by PCR, fragmented, and cotransfected or electorporated with a vector into which reassembled V region genes can recombine. DNA substrate molecules are recovered from the cotranfected cells, and the process is repeated until the desired level of improvment is obtained. Other embodiments include reassembling the V regions prior to the electroporation so that an intact V region exon can recombine into an antibody expression cassette. Further embodiments include the use of this format for other eukaryotic genes or for the evolution of whole viruses.

G. **Phagemid-Based Assembly**

In some embodiments of the invention, a gene of interest is cloned into a vector that generates single stranded DNA, such as a phagemid. The resulting DNA substrate is mutagenzied by RSR in any method known in the art, transfected into host cells, and subjected to a screen or selection for a desired property or improved phenotype. DNA from the selected or screened phagemids is amplified, by, for example, PCR or plasmid preparation. This DNA preparation contains the various mutant sequences that one wishes to permute. This DNA is fragmented and denatured, and annealed with single-stranded DNA (ssDNA) phagemid template (ssDNA encoding the wild-type gene and vector sequences). A preferred embodiment is the use of dut(-) ung(-) host strains such as CJ236 (Sambrook et al., Molecular Cloning CSH Press (1987)) for the preparation of ssDNA.

Gaps in annealed template are filled with DNA polymerase and ligated to form closed relaxed circles. Since multiple fragments can anneal to the phagemid, the newly synthesized strand now consists of shuffled sequences. These products are transformed into a mutS strain of *E. coli* which is dut+ ung+. Phagemid DNA is recovered from the transfected host and subjected again to this protocol until the desired level of improvement is obtained. The gene encoding the protein of interest in this library of recovered phagemid DNA can be mutagenzied by any technique, including RSR, before the process is repeated.

III. **Improved Protein Expression**

While recombinant DNA technology has proved to be a very general method for obtaining large, pure, and homogeneous quantities of almost all nucleic acid sequences of interest, similar generality has not yet been achieved for the production of large amounts

of pure, homogeneous protein in recombinant form. A likely explanation is that protein expression, folding, localization and stability is intrinsically more complex and unpredictable than for DNA. The yield of expressed protein is a complex function of transcription rates, translation rates, interactions with the ribosome, interaction of the nascent polypeptide with chaperonins and other proteins in the cell, efficiency of oligomerization, interaction with components of secretion and other protein trafficking pathways, protease sensitivity, and the intrinsic stability of the final folded state. Optimization of such complex processes is well suited for the application of RSR. The following methods detail strategies for application of RSR to the optimization of protein expression.

A. Evolution of Mutant Genes with Improved Expression Using RSR on Codon Usage Libraries

The negative effect of rare *E. coli* codons on expression of recombinant proteins in this host has been clearly demonstrated (Rosenberg, et al., J. Bact. 175:716-722 (1993)). However, general rules for the choice of codon usage patterns to optimize expression of functional protein have been elusive. In some embodiments of the invention, protein expression is optimized by changing codons used in the gene of interest, based on the degeneracy of the genetic code. Typically, this is accomplished by synthesizing the gene using degenerate oligonucleotides. In some embodiments the degenerate oligonucleotides have the general structure of about 20 nucleotides of identity to a DNA substrate molecule encoding a protein of interest, followed by a region of about 20 degenerate nucleotides which encode a region of the protein, followed by another region of about 20 nucleotides of identity. In a preferred embodiment, the region of identity utilizes preferred codons for the host. In a further embodiment, the oligonucleotides are identical to the DNA substrate at least one 5' and one 3' nucleotide, but have at least 85% sequence homology to the DNA substrate molecule, with the difference due to the use of degenerate codons. In some embodiments, a set of such degenerate oligonucleotides is used in which each oligonucleotide overlaps with another by the general formula n - 10, wherein n is the length of the oligonucleotide. Such oligonucleotides are typically about 20 - 1000 nucleotides in length. The assembled genes are then cloned, expressed, and screened or selected for improved expression. The assembled genes can be subjected to recursive recombination methods as descibed above until the desired improvement is achieved.

For example, this technique can be used to evolve bovine intestinal alkaline phosphatase (BIAP) for active expression in E. coli. This enzyme is commonly used as a reporter gene in assay formats such as ELISA. The cloned gene cannot be expressed in active form in a prokaryotic host such as *E. coli* in good yield. Development of such an expression system would allow one to access inexpensive expression technology for BIAP

and, importantly, for engineered variants with improved activity or chemical coupling properties (such as chemical coupling to antibodies). A detailed example is provide in the Experimental Examples section.

B. Improved Folding

In some embodiments of the invention, proteins of interest when overexpressed or expressed in heterologous hosts form inclusion bodies, with the majority of the expressed protein being found in insoluble aggregates. Recursive sequence recombination techniques can be used to optimize folding of such target proteins. There are several ways to improve folding, including mutating evolving the target protein of interest and evolving chaperonin proteins.

1. Evolving A Target Protein

a. Inclusion Body Fractionation Selection Using lac Headpiece Dimer Fusion Protein

The lac repressor "headpiece dimer" is a small protein containing two headpiece domains connected by a short peptide linker which binds the lac operator with sufficient affinity that polypeptide fusions to this headpiece dimer will remain bound to the plasmid that encodes them throughout an affinity purification process (Gates et al., J. Mol. Biol. 255:373-386 (1995)). This property can be exploited, as follows, to evolve mutant proteins of interest with improved folding properties. The protein of interest can be mammalian, yeast, bacterial, etc.

A fusion protein between the lac headpiece dimer and a target protein sequence is constructed, for example, as disclosed by Gates (supra). This construct, containing at least one lac operator, is mutagenized by technologies common in the arts such as PCR mutagenesis, chemical mutagenesis, oligo directed mutagenesis (Sambrook et al., Molecular Cloning CSH Press (1987)). The resulting library is transformed into a host cell, and expression of the fusion protein is induced, preferably with arabinose. An extract or lysate is generated from a culture of the library expressing the construct. Insoluble protein is fractionated from soluble protein/DNA complexes by centrifugation or affinity chromatography, and the yield of soluble protein/DNA complexes is quantitated by quantitative PCR (Sambrook et al., Molecular Cloning, CSH Press, 1987) of the plasmid. Preferably, a reagent that is specific for properly folded protein, such as a monoclonal antibody or a natural ligand, is used to purify soluble protein/DNA complexes. The plasmid DNA from this step is isolated, subjected to RSR and again expressed. These steps are repeated until the yield of soluble protein/DNA complexes has reached a desired level of improvement. Individual clones are then screened for retention of functional properties of the protein of interest, such as enzymatic activity, etc.

This technique is generically useful for evolving solubility and other properties such as cellular trafficking of proteins heterologously expressed in a host cell of interest. For example, one could select for efficient folding and nuclear localization of a protein fused to the lac repressor headpiece dimer by encoding the protein on a plasmid encoding an SV40 origin of replication and a lac operator, and transiently expressing the fusion protein in a mammalian host expressing T antigen. Purification of protein/DNA complexes from nuclear HIRT extracts (Seed and Aruffo, Proc. Natl. Acad. Sci. (U.S.A.), 84:3365-3369 (1987)) would allow one to select for efficient folding and nuclear localization proteins.

b. Functional Expression of Protein Using Phage Display

A problem often encountered in phage display methods such as those disclosed by O'Neil et al. (Current Biology, 5:443-449 (1995)) is the inability to functionally express a protein of interest on phage. Without being limited to any one theory, improper folding of the protein of interest can be responsible for this problem. RSR can be used to evolve a protein of interest for functional expression on phage. Typically, a fusion protein is constructed between gene III or gene VIII and the target protein and then mutagenized, for example by PCR mutagenesis. The mutagenzied library is then expressed in a phage display format, a phage stock is made, and these phage are affinity selected for those bearing functionally displayed fusion proteins using an affinity matrix containing a known ligand for the target protein. DNA from the functionally selected phage is purified, and the displayed genes of interest are shuffled and recloned into the phage display format. The selection, shuffling and recloning steps are repeated until the yield of phage with functional displayed protein has reached desired levels as defined, for example, by the fraction of phage that are retained on a ligand affinity matrix or the biological activity associated with the displayed phage. Individual clones are then screened to identify candidate mutants with improved display properties, desired level of expression, and functional properties of interest (e.g., ability to bind a ligand or receptor, lymphokine activity, enzymatic activity, etc.).

In some embodiments of the invention, a functional screen or selection is used to identify an evolved protein not expressed on a phage. The target protein, which cannot initially be efficiently expressed in a host of interest, is mutagenized and a functional screen or selection is used to identify cells expressing functional protein. For example, the protein of interest may complement a function in the host cell, cleave a colorimetric substrate, etc. Recursive sequence recombination is then used to rapidly evolve improved functional expression from such a pool of improved mutants.

For example, AMV reverse transcriptase is of particular commercial importance because it is active at a higher temperature (42° C) and is more robust than many other reverse transcriptases. However, it is difficult to express in prokaryotic hosts

such as *E. coli*, and is consequently expensive because it has to be purified from chicken cells. Thus an evolved AMV reverse transcriptase that can be expressed efficiently in *E. coli* is highly desirable.

In brief, the AMV reverse transcriptase gene (Papas et al., J. Cellular Biochem 20:95-103 (1982)) is mutagenized by any method common in the art. The library of mutant genes is cloned into a colE1 plasmid (Amp resistant) under control of the lac promoter in a polA12 (Ts) recA718 (Sweasy et al. Proc. Natl. Acad. Sci. U.S.A. 90:4626-4630 (1993)) E. coli host. The library is induced with IPTG, and shifted to the nonpermissive temperature. This selects for functionally expressed reverse transcriptase genes under the selective conditions reported for selection of active HIV reverse transcriptase mutants reported by Kim et al. (Proc. Natl. Acad. Sci. (U.S.A.), 92:684-688 (1995)). The selected AMV RTX genes are recovered by PCR by using oligonucleotides flanking the cloned gene. The resulting PCR products are subjected to in vitro RSR, selected as described above, and the process is repeated until the level of functional expression is acceptable. Individual clones are then screened for RNA-dependent DNA polymerization and other properties of interest (e.g. half life at room temperature, error rate). The candidate clones are subjected to mutagenesis, and then tested again to yield an AMV RT that can be expressed in *E. coli* at high levels.

2. **Evolved Chaperonins**

In some embodiments of the invention, overexpression of a protein can lead to the accumulation of folding intermediates which have a tendency to aggregate. Without being limited to any one theory, the role of chaperonins is thought to be to stabilize such folding intermediates against aggregration; thus, overexpression of a protein of interest can lead to overwhelming the capacity of chaperonins. Chaperonin genes can be evolved using the techniques of the invention, either alone or in combination with the genes encoding the protein of interest, to overcome this problem.

Examples of proteins of interest which are especially suited to this approach include but are not limited to: cytokines; malarial coat proteins; T cell receptors; antibodies; industrial enzymes (e.g., detergent proteases and detergent lipases); viral proteins for use in vaccines; and plant seed storage proteins.

Sources of chaperonin genes include but are limited to *E. coli* chaperonin genes encoding such proteins as thioredoxin, Gro ES/Gro EL, PapD, ClpB, DsbA, DsbB, DnaJ, DnaK, and GrpE; mammalian chaperonins such as Hsp70, Hsp72, Hsp73, Hsp40,Hsp60, Hsp10, Hdj1, TCP-1, Cpn60, BiP; and the homologues of these chaperonin genes in other species such as yeast (J.G. Wall and A. Pluckthun, Current Biology, 6:507-

516 (1995); Hartl, Nature, 381:571-580 (1996)). Additionally, heterologous genomic or cDNA libraries can be used as libraries to select or screen for novel chaperonins.

In general, evolution of chaperonins is accomplished by first mutagenizing chaperonin genes, screening or selecting for improved expression of the target protein of interest, subjecting the mutated chaperonin genes to RSR, and repeating selection or screening. As with all RSR techniques, this is repeated until the desired improvement of expression of the protein of interest is obtained. Two exemplary approaches are provide below.

a. Chaperonin Evolution in Trans to the Protein of Interest With a Screen or Selection for Improved Function

In some embodiments the chaperonin genes are evolved independently of the gene(s) for the protein of interest. The improvement in the evolved chaperonin can be assayed, for example, by screening for enhancement of the activity of the target protein itself or for the activity of a fusion protein comprising the target protein and a selectable or screenable protein (e.g., GFP, alkaline phosphatase or beta-galactosidase).

b. Chaperonin Operon in cis

In some embodiments, the chaperonin genes and the target protein genes are encoded on the same plasmid, but not necessarily evolved together. For example, a lac headpiece dimer can be fused to the protein target to allow for selection of plasmids which encode soluble protein. Chaperonin genes are provided on this same plasmid ("cis") and are shuffled and evolved rather than the target protein. Similarly, the chaperonin genes can be cloned onto a phagemid plasmid that encodes a gene III or gene VIII fusion with a protein of interest. The cloned chaperonins are mutagenized and, as with the selection described above, phage expressing functionally displayed fusion protein are isolated on an affinity matrix. The chaperonin genes from these phage are shuffled and the cycle of selection, mutation and recombination are applied recursively until fusion proteins are efficiently displayed in functional form.

3. Improved Intracellular Localization

Many overexpressed proteins of biotechnological interest are secreted into the periplasm or media to give advantages in purification or activity assays. Optimization for high level secretion is difficult because the process is controlled by many genes and hence optimization may require multiple mutations affecting the expression level and structure of several of these components. Protein secretion in *E. coli*, for example, is known to be influenced by many proteins including: a secretory ATPase (SecA), a translocase complex (SecB, SecD, SecE, SecF, and SecY), chaperonins (DnaK, DnaJ, GroES, GroEL), signal peptidases (LepB, LspA, Ppp), specific folding catalysts (DsbA) and other proteins of less

well defined function (e.g., Ffh, FtsY) (Sandkvist et al., Curr. Op. Biotechnol. 7:505-511 (1996)). Overproduction of wild type or mutant copies of these genes for these proteins can significantly increase the yield of mature secreted protein. For example, overexpression of secY or secY4 significantly increased the periplasmic yield of mature human IL6 from a hIL6-pre-OmpA fusion (Perez-Perez et al., Bio-Technology 12:178-180 (1994)). Analogously, overexpression of DnaK/DnaJ in *E. coli* improved the yield of secreted human granulocyte colony stimulating factor (Perez-Perez et al., Biochem. Biophys. Res. Commun. 210:254-259 (1995)).

RSR provides a route to evolution of one or more of the above named components of the secretory pathway. The following strategy is employed to optimize protein secretion in *E. coli*. Variations on this method, suitable for application to *Bacillus subtilis*, *Pseudomonas*, *Saccaromyces cerevisiæ*, *Pichia pastoris*, mammalian cells and other hosts are also described. The general protocol is as follows.

One or more of the genes named above are obtained by PCR amplification from *E. coli* genomic DNA using known flanking sequence, and cloned in an ordered array into a plasmid or cosmid vector. These genes do not in general occur naturally in clusters, and hence these will comprise artificial gene clusters. The genes may be cloned under the control of their natural promoter or under the control of another promoter such as the lac, tac, arabinose, or trp promoters. Typically, rare restriction sites such as Sfi I are placed between the genes to facilitate ordered reassembly of shuffled genes as described in the methods of the invention.

The gene cluster is mutagenized and introduced into a host cell in which the gene of interest can be inducibly expressed. Expression of the target gene to be secreted and of the cloned genes is induced by standard methods for the promoter of interest (e.g., addition of 1 mM IPTG for the lac promoter). The efficiency of protein secretion by a library of mutants is measured, for example by the method of colony blotting (Skerra et al., Anal. Biochem. 196:151-155 (1991)). Those colonies expressing the highest levels of secreted protein (the top 0.1 - 10%; preferably the top 1%) are picked. Plasmid DNA is prepared from these colonies and shuffled according to any of the methods of the invention.

Preferably, each individual gene is amplified from the population and subjected to RSR. The fragments are digested with Sfi I (introduced between each gene with nonpalindromic overhangs designed to promote ordered reassembly by DNA ligase) and ligated together, preferably at low dilution to promote formation of covalently closed relaxed circles (<1 ng/microliter). Each of the PCR amplified gene populations may be shuffled prior to reassembly into the final gene cluster. The ligation products are transformed back into the host of interest and the cycle of selection and RSR is repeated.

Analogous strategies can be employed in other hosts such as *Pseudomonas*, *Bacillus subtilis*, yeast and mammalian cells. The homologs of the *E. coli* genes listed above are targets for optimization, and indeed many of these homologs have been identified in other species (Pugsley, Microb. Rev. 57:50-108 (1993)). In addition to these homologs, other components such as the six polypeptides of the signal recognition particle, the translocating chain-associating membrane protein (TRAM), BiP, the Ssa proteins and other hsp70 homologs, and prsA (*B. subtilis*) (Simonen and Pulva, Microb. Rev. 57:109-137 (1993)) are targets for optimization by RSR. In general, replicating episomal vectors such as SV40-neo (Sambrook et al., Molecular Cloning, CSH Press (1987), Northrup et al., J. Biol. Chem. 268(4):2917-2923 (1993)) for mammalian cells or 2 micron or ars plasmids for yeast (Strathern et al., The Molecular Biology of the Yeast Saccaromyces, CSH Press (1982)) are used. Integrative vectors such as pJM 103, pJM 113 or pSGMU2 are preferred for *B. subtilis* (Perego, Chap. 42, pp. 615-624 in: *Bacillus subtilis* and Other Gram-Positive Bacteria, A. Sonenshein, J. Hoch, and R. Losick, eds., 1993).

For example, an efficiently secreted thermostable DNA polymerase can be evolved, thus allowing the performance of DNA polymerization assays with little or no purification of the expressed DNA polymerase. Such a procedure would be preferred for the expression of libraries of mutants of any protein that one wished to test in a high throughput assay, for example any of the pharmaceutical proteins listed in Table I, or any industrial enzyme. Initial constructs are made by fusing a signal peptide such as that from STII or OmpA to the amino terminus of the protein to be secreted. A gene cluster of cloned genes believed to act in the secretory pathway of interest are mutagenized and coexpressed with the target construct. Individual clones are screened for expresion of the gene product. The secretory gene clusters from improved clones are recovered and recloned and introduced back into the original host. Preferably, they are first subjected to mutagenesis before the process is repeated. This cycle is repeated until the desired improvement in expression of secreted protein is achieved.

IV. Evolved Polypeptide Properties

A. Evolved Transition State Analog and Substrate Binding

There are many enzymes of industrial interest that have substantially suboptimal activity on the substrate of interest. In many of these cases, the enzyme obtained from nature is required to work either under conditions that are very different from the conditions under which it evolved or to have activity towards a substrate that is different from the natural substrate.

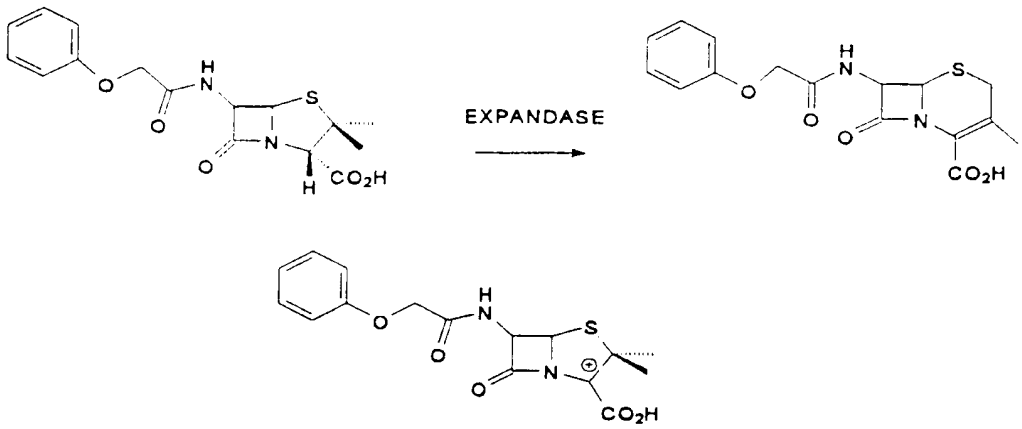
The application of evolutionary technologies to industrial enzymes is often significantly limited by the types of selections that can be applied and the modest numbers of

mutants that can be surveyed in screens. Selection of enzymes or catalytic antibodies, expressed in a display format, for binding to transition state analogs (McCafferty et al., Appl. Biochem. Biotechnol. 47:157-171 (1994)) or substrate analogs (Janda et al., Proc. Natl. Acad. Sci. (U.S.A.) 91:2532-2536, (1994)) represents a general strategy for selecting for mutants with with improved catalytic efficiency.

Phage display (O'Neil et al., Current Biology 5:443-449 (1995) and the other display formats (Gates et al., J. Mol. Biol. 255:373-386 (1995); Mattheakis et al., Proc. Natl. Acad. Sci. (U.S.A.) 91:9022-9026 (1994)) described herein represent general methodologies for applying affinity-based selections to proteins of interest. For example, Matthews and Wells (Science 260:1113-1117 (1993)) have used phage display of a protease substrate to select improved substrates. Display of active enzymes on the surface of phage, on the other hand, allows selection of mutant proteins with improved transition state analog binding. Improvements in affinity for transition state analogs correlate with improvements in catalytic efficiency. For example, Patten et al., Science 271:1086-1091 (1996) have shown that improvements in affinity of a catalytic antibody for its hapten are well correlated with improvements in catalytic efficiency, with an 80-fold improvement in kcat/Km being achieved for an esterolytic antibody.

For example, an enzyme used in antibiotic biosynthesis can be evolved for new substrate specificity and activity under desired conditions using phage display selections. Some antibiotics are currently made by chemical modifications of biologically produced starting compounds. Complete biosynthesis of the desired molecules is currently impractical because of the lack of an enzyme with the required enzymatic activity and substrate specificity (Skatrud, TIBTECH 10:324-329, September 1992). For example, 7-aminodeacetooxycephalosporanic acid (7-ADCA) is a precursor for semi-synthetically produced cephalosporins. 7-ADCA is made by a chemical ring expansion of penicillin G followed by enzymatic deacylation of the phenoxyacetal group. 7-ADCA can be made enzymatically from deacetylcephalosporin C (DAOC V), which could in turn be derived from penicillin V by enzymatic ring expansion if a suitably modified penicillin expandase could be evolved (Cantwell et al., Curr. Genet. 17:213-221 (1990)). Thus, 7-ADCA could in principle be produced enzymatically from penicillin V using a modified penicillin N expandase, such as mutant forms of the *S. clavuligerus* cefE gene (Skatrud, TIBTECH 10:324-329, September 1992). However, penicillin V is not accepted as a substrate by any known expandase with sufficient efficiency to be commercially useful. As outlined below, RSR techniques of the invention can be used to evolve the penicillin expandase encoded by cefE or other expandases so that they will use penicillin V as a substrate.

Phage display or other display format selections are applied to this problem by expressing libraries of cefE penicillin expandase mutants in a display format, selecting for binding to substrates or transition state analogs, and applying RSR to rapidly evolve high affinity binders. Candidates are further screened to identify mutants with improved enzymatic activity on penicillin V under desired reaction conditions, such as pH, temperature, solvent concentration, etc. RSR is applied to further evolve mutants with the desired expandase activity. A number of transition state analogs (TSA's) are suitable for this reaction. The following structure is the initial TSA that is used for selection of the display library of cefE mutants:



Libraries of the known penicillin expandases (Skatrud, TIBTECH 10:324-329(1992); Cantwell et al., Curr. Genet. 17:13-221 (1990)) are made as described herein. The display library is subjected to selection for binding to penicillin V and/or to transition state analog given above for the conversion of penicillin V to DAOC V. These binding selections may be performed under non-physiological reaction conditions, such as elevated temperature, to obtain mutants that are active under the new conditions. RSR is applied to evolve mutants with 2 - 10^5 fold improvement in binding affinity for the selecting ligand. When the desired level of improved binding has been obtained, candidate mutants are expressed in a high throughput format and specific activity for expanding penicillin V to DAOC V is quantitatively measured. Recombinants with improved enzymatic activity are mutagenized and the process repeated to further evolve them.

Retention of TSA binding by a displayed enzyme (e.g., phage display, lac headpiece dimer, polysome display, etc.) is a good selection for retention of the overall integrity of the active site and hence can be exploited to select for mutants which retain activity under conditions of interest. Such conditions include but are not limited to: different pH optima, broader pH optima, activity in altered solvents such as DMSO (Seto et al., DNA

Sequence 5:131-140 (1995)) or formamide (Chen et al., Proc. Natl. Acad. Sci. (U.S.A.) 90:5618-5622, (1993)) altered temperature, improved shelf life, altered or broadened substrate specificity, or protease resistance. A further example, the evolution of a p-nitrophenyl esterase, using a mammalian display format, is provided below.

B. Improvement of DNA and RNA Polymerases

Of particular commercial importance are improved polymerases for use in nucleic acid sequencing and polymerase chain reactions. The following properties are attractive candidates for improvement of a DNA sequencing polymerase: (1) suppression of termination by inosine in labelled primer format (H. Dierick et al., Nucleic Acids Res. 21:4427-4428 (1993)) (2) more normalized peak heights, especially with fluorescently labelled dideoxy terminators (Parker et al., BioTechniques 19:116-121 (1995)), (3) better sequencing of high GC content DNA (>60% GC) by, for example, tolerating >10% DMSO (D. Seto et al., DNA Sequence 5:131-140 (1995); Scheidl et al., BioTechniques 19(5):691-694 (1995)), or (4) improved acceptance of novel base analogs such as inosine, 7-deaza dGTP (Dierick et al., Nucleic Acids Res. 21:4427-4428 (1993)) or other novel base analogs that improve the above properties.

Novel sequencing formats have been described which use matrix assisted laser desorption ionization time of flight (MALDT-TOF) mass spectroscopy to resolve dideoxy ladders (Smith, Nature Biotechnology 14:1084-1085 (1996)). It is noted in Smith's recent review that fragmentation of the DNA is the singular feature limiting the development of this method as a viable alternative to standard gel electrophoresis for DNA sequencing. Base analogs which stabilize the N-glycosidic bond by modifications of the purine bases to 7-deaza analogs (Kirpekar et al., Rapid Comm. in Mass Spec. 9:525-531 (1995)) or of the 2' hydroxyl (such as 2'-H or 2'-F) "relieve greatly the mass range limitation" of this technique (Smith, 1996). Thus, evolved polymerases that can efficiently incorporate these and other base analogs conferring resistance to fragmentation under MALDI-TOF conditions are valuable innovations.

Other polymerase properties of interest for improvement by RSR are low fidelity thermostable DNA polymerase for more efficient mutagenesis or as a useful correlate for acceptance of base analogs for the purposes described above; higher fidelity polymerase for PCR (Lundberg et al., Gene 108:1-6 (1991)); higher fidelity reverse transcriptase for retroviral gene therapy vehicles to reduce mutation of the therapeutic construct and of the retrovirus; improved PCR of GC rich DNA and PCR with modified bases (S. Turner and F. J. Jenkins, BioTechniques 19(1):48-52 (1995)).

Thus, in some embodiments of the invention, libraries of mutant polymerase genes are screened by direct high throughput screening for improved sequencing properties.

The best candidates are then subjected to RSR. Briefly, mutant libraries of candidate polymerases such as Taq polymerase are constructed using standard methods such as PCR mutagenesis (Caldwell et al., PCR Meth. App. 2:28-33 (1992)) and/or cassette mutagenesis (Sambrook et al., Molecular Cloning, CSH Press (1987)). Incorporation of mutations into Taq DNA polymerase such as the active site residue from T7 polymerase that improves acceptance of dideoxy nucleotides (Tabor and Richardson, J. Biol. Chem. 265:8322-8328 (1990)) and mutations that inactivate the 5' - 3' exonuclease activity (R.S. Rano, BioTechniques 18:390-396 (1995)) are incorporated into these libraries. The reassembly PCR technique, for example, as described above is especially suitable for this problem. Similarly, chimeric polymerase libraries are made by breeding existing thermophilic polymerases, sequenase, and *E. coli* poll with each other using the bridge oligonucleotide methods described above. The libraries are expressed in formats wherein human or robotic colony picking is used to replica pick individual colonies into 96 well plates where small cultures are grown, and polymerase expression is induced.

A high throughput, small scale simple purification for polymerase expressed in each well is performed. For example, simple single-step purifications of His-tagged Taq expressed in *E. coli* have been described (Smirnov et al., Russian J. Bioorganic Chem. 21(5):341-342 (1995)), and could readily be adapted for a 96-well expression and purification format.

A high throughput sequencing assay is used to perform sequencing reactions with the purified samples. The data is analyzed to identify mutants with improved sequencing properties, according to any of these criteria: higher quality ladders on GC-rich templates, especially greater than 60% GC, including such points as fewer artifactual termination products and stronger signals than given with the wild-type enzyme; less termination of reactions by inosine in primer labelled reactions, e.g., fluorescent labelled primers; less variation in incorporation of signals in reactions with fluorescent dideoxy nucleotides at any given position; longer sequencing ladders than obtained with the wild-type enzyme, such as about 20 to 100 nucleotides; improved acceptance of other known base analogs such as 7-deaza purines;
improved acceptance of new base analogs from combinatorial chemistry libraries (See, for example, Hogan, Nature 384(Supp):17-1996).

The best candidates are then subjected to mutagenesis, and then selected or screened for the improved sequencing properties decribed above.

In another embodiment, a screen or selection is performed as follows. The replication of a plasmid can be placed under obligate control of a polymerase expressed in *E. coli* or another microorganism. The effectiveness of this system has been demonstrated for

making plasmid replication dependent on mammalian polymerase beta (Sweasy et al., Proc. Natl. Acad. Sci. (U.S.A.) 90:4626-4630, (1993)), Taq polymerase (Suzuki et al., Proc. Natl. Acad. Sci. (U.S.A.) 93:9670-9675 (1996)), or HIV reverse transcriptase (Kim et al., Proc. Natl. Acad. Sci. (U.S.A.) 92:684-688 (1995)). The mutant polymerase gene is placed on a plasmid bearing a colE1 origin and expressed under the control of an arabinose promoter. The library is enriched for active polymerases essentially as described by Suzuki et al., (supra), with polymerase expression being induced by the presence of arabinose in the culture.

A further quantitative screen utilizes the presence of GFP (green fluorescence protein) on the same plasmid, replica plating onto arabinose at the nonpermissive temperature in the absence of a selective antibiotic, and using a fluorimeter to quantitatively measure fluorescence of each culture. GFP activity correlates with plasmid stability and copy number which is in turn dependent on expression of active polymerase.

A polymerase with a very high error rate would be a superior sequencing enzyme, as it would have a more normalized signal for incorporation of base analogs such as the currently used fluorescently labelled dideoxies because it will have reduced specificity and selectivity. The error rates of currently used polymerases are on the order of 10^{-5} to 10^{-6}, orders of magnitude lower than what can be detected given the resolving power of the gel systems. An error rate of 1%, and possibly as high as 10%, could not be detected by current gel systems, and thus there is a large window of opportunity to increase the "sloppiness" of the enzyme. An error-prone cycling polymerase would have other uses such as for hypermutagenesis of genes by PCR.

In some embodiments, the system described by Suzuki (Suzuki et al., Proc. Natl. Acad. Sci. (U.S.A.) 96:9670-9675 (1996)) is used to make replication of a reporter plasmid dependent on the expressed polymerase. This system puts replication of the first 200 - 300 bases next to the ColE1 origin directly under the control of the expressed polymerase (Sweasy and Loeb, J. Bact. 177:2923-2925 (1995); Sweasy et al., Proc. Natl. Acad. Sci. (U.S.A.) 90:4626-4630 (1993)). A screenable or selectable reporter gene containing stop codons is positioned in this region, such as LacZ alpha containing one, two or three stop codons. The constructs are grown on arabinose at the nonpermissible temperature, allowed to recover, and plated on selective lactose minimal media that demands reversion of the stop codons in the reporter cassette. Mutant polymerases are recovered from the survivors by PCR. The survivors are enriched for mutators because their mutator phenotype increases the rate of reversion of stop codons in the reporter lacZ alpha fragment.

The polymerase genes from the survivors are subjected to RSR, then the polymerase mutants are retransformed into the indicator strain. Mutators can be visually

screened by plating on arabinose/Xgal plates at the nonpermissive temperature. Mutator polymerases will give rise to colonies with a high frequency of blue papillae due to reversion of the stop codon(s). Candidate papillators can be rescreened by picking a non-papillating region of the most heavily papillated colonies (i.e, "best" colonies) and replating on the arabinose/Xgal indicator medium to further screen for colonies with increased papillation rates. These steps are repeated until a desired reversion rate is achieved (e.g., 10^{-2} to 10^{-3} mutations per base pair per replication).

Colonies which exhibit high frequency papillation are candidates for encoding an error prone polymerase. These candidates are screened for improved sequencing properties essentially as for the high throughput screen described above. Briefly, mutant Taq proteins are expressed and purified in a 96-well format. The purified proteins are used in sequencing reactions and the sequence data are analyzed to identify mutants that exhibit the improvements outlined herein. Mutants with improved properties are subjected to RSR and rescreened for further improvements in function.

In some embodiments, GFP containing stop codons instead of lacZ alpha with stop codons is used for the construction. Cells with reverted stop codons in GFP are selected by fluorescence activated cell sorter (FACS). In general, FACS selection is performed by gating the brightest about 0.1- 10%, preferably the top 0.1 to 1%, and collected according to a protocol similar to that of Dangl et al., (Cytometry 2(6):395-401 (1982)). In other embodiments, the polA gene is flanked with lox sites or other targets of a site specific recombinase. The recombinase is induced, thus allowing one to inducibly delete the polA gene (Mulbery et al., Nucleic Acid Res. 23:485-490 (1995)). This would allow one to perform "Loeb-type" selections at any temperature and in any host. For example, one could set up such a selection in a recA deficient mesophile or thermophile by placing the polA homologue in an inducibly deletable format and thus apply the selection for active polymerase under more general conditions.

In further embodiments, this general system is preferred for directed in vivo mutagenesis of genes. The target gene is cloned into the region near a plasmid origin of replication that puts its replication obligately under control of the error prone polymerase. The construct is passaged through a polA(ts) recA strain and grown at the nonpermissive temperature, thus specifically mutagenizing the target gene while replicating the rest of the plasmid with high fidelity.

In other embodiments, selection is based on the ability of mutant DNA polymerases to PCR amplify DNA under altered conditions or by utilizing base analogs. The mutant polymerases act on the template that encodes them in a PCR amplification, thus differentially replicating those polymerases.

In brief, an initial library of mutants is replica plated. Polymerase preparations are done in a 96-well format. Crude plasmid preparations are made of the same set. Each plasmid prep is PCR-amplified using the polymerase prep derived from that plasmid under the conditions for which one wishes to optimize the polymerase (e.g., added DMSO or formamide, altered temperature of denaturation or extension, altered buffer salts, PCR with base analogs such a-thiol dNTP's for use with mass spectroscopy sequencing, PCR of GC rich DNA (>60% GC),PCR with novel base analogs such as 7-deaza purines, 2' fluoro dNTP's, rNTP's, PCR with inosine, etc.). The amplified genes are pooled, cloned,and subjected to mutagenesis, and the process repeated until an improvement is achieved.

C. **Evolved Phosphonatase**

Alkaline phosphatase is a widely used reporter enzyme for ELISA assays, protein fusion assays, and in a secreted form as a reporter gene for mammalian cells. The chemical lability of p-nitrophenyl phosphate (pNPP) substrates and the existence of cellular phosphatases that cross-react with pNPP is an important limitation on the sensitivity of assays using this reporter gene. A reporter gene with superior signal to noise properties can be developed based on hydrolysis of p-nitrophenyl phosphonates, which are far more stable to base catalyzed hydrolysis than the corresponding phosphates. Additionally, there are far fewer naturally occurring cellular phosphonatases than alkaline phosphatases. Thus a p-nitrophenyl phosphonatase is an attractive replacement for alkaline phosphatase because the background due to chemical and enzymatic hydrolysis is much lower. This will allow one to make ELISA's more sensitive for detecting very small concentrations of antigen.

Chen et al. (J. Mol. Biol. 234:165-178 (1993)) have shown that a *Staph. aureus* beta-lactamase can hydrolyze p-nitrophenyl phosphonate esters with single turnover kinetics. The active site Ser70 (the active site nucleophile for beta lactam hydrolysis) forms a covalent intermediate with the substrate. This is analogous to the first step in hydrolysis of beta lactams, and this enzyme can be evolved by RSR to hydrolyze phosphonates by a mechanism analogous to beta lactam hydrolysis. Metcalf and Wanner have described a cryptic phosphonate utilizing operon (phn) in *E. coli*, and have constructed strains bearing deletions of the phn operon (J. Bact. 175:3430-3442 (1993)). This paper discloses selections for growth of *E. coli* on phosphate free minimal media where the phosphorous is derived from hydrolysis of alkyl phosphonates by genes in the phn operon. Thus, one could select for evolved p-nitrophenyl phosphonatases that are active using biochemical selections on defined minimal media. Specifically, an efficient phosphonatase is evolved as follows. A library of mutants of the *Staph. aureus* beta lactamase or of one of the *E. coli* phn enzymes is constructed. The library is transformed into *E. coli* mutants wherein the phn operon has been deleted, and selected for growth on phosphate free MOPS minimal media containing p-

nitrophenyl phosphonate. RSR is applied to selected mutants to further evolve the enzyme for improved hydrolysis of p-nitrophenyl phosphonates.

D. Evolved Detergent Proteases

Proteases and lipases are added in large quantities to detergents to enzymatically degrade protein and lipid stains on clothes. The incorporation of these enzymes into detergents has significantly reduced the need for surfactants in detergents with a consequent reduction in the cost of formulation of detergents and improvement in stain removal properties. Proteases with improved specific activity, improved range of protein substrate specificity, improved shelf life, improved stability at elevated temperature, and reduced requirements for surfactants would add value to these products.

As an example, subtilisin can be evolved as follows. The cloned subtilisin gene (von der Osten et al., J. Biotechnol. 28:55-68 (1993)) can be subjected to RSR using growth selections on complex protein media by virtue of secreted subtilisin degrading the complex protein mixture. More specifically, libraries of subtilisin mutants are constructed in an expression vector which directs the mutant protein to be secreted by *Bacillus subtilus*. Bacillus hosts transformed with the libraries are grown in minimal media with complex protein formulation as carbon and/or nitrogen source. Subtilisin genes are recovered from fast growers and subjected to RSR, then screened for improvement in a desired property.

E. Escape of Phage from a "Protein Net"

In some embodiments, selection for improved proteases is performed as follows. A library of mutant protease genes is constructed on a display phage and the phage grown in a multiwell format or on plates. The phage are overlayed with a "protein net" which ensnares the phage. The net can consist of a protein or proteins engineered with surface disulphides and then crosslinked with a library of peptide linkers. A further embodiment employs an auxiliary matrix to further trap the phage. The phage are further incubated, then washed to collect liberated phage wherein the displayed protease was able to liberate the phage from the protein net. The protease genes are then subjected to RSR for further evolution. A further embodiment employs a library of proteases encoded by but not displayed on a phagemid wherein streptavidin is fused to pIII by a peptide linker. The library of protease mutants is evolved to cleave the linker by selecting phagemids on a biotin column between rounds of amplification.

In a further embodiment, the protease is not necessarily provided in a display format. The host cells secrete the protease encoded by but not surface diplayed by a phagemid, while constrained to a well, for example, in a microtiter plate. Phage display format is preferred where an entire high titre lysate is encased in a protein net matrix, and

the phage expressing active and broad specificity proteases digesting the matrix to be liberated for the next round of amplification, mutagenesis, and selection.

In a further embodiment, the phage are not constrained to a well but, rather, protein binding filters are used to make a colony of plaque lifts and are screened for activity with chromogenic or fluorogenic substrates. Colonies or plaques corresponding to positive spots on the filters are picked and the encoded protease genes are recovered by, for example, PCR. The protease genes are then subjected to RSR for further evolution.

F. Screens for Improved Protease Activity

Peptide substrates containing fluorophores attached to the carboxy terminus and fluorescence quenching moities on the amino terminus, such as those described by Holskin, et al, (Anal. Biochem. 227:148-55 (1995)) (e.g., (4-4'-dimethylaminophenazo)benzoyl-arg-gly-val-val-asn-ala-ser-ser-arg-leu-ala-5-(2'-aminoethyl)-amino-naphthalene-1-sulfonic acid) are used to screen protease mutants for broadened or altered specificity. In brief, a library of peptide substrates is designed with a flourophore on the amino terminus and a potent fluorescence quencher on the carboxy terminus, or vice versa. Supernatants containing secreted proteases are incubated either separately with various members of the library or with a complex cocktail. Those proteases which are highly active and have broad specificity will cleave the majority of the peptides, thus releasing the fluorophore from the quencher and giving a positive signal on a fluorimeter. This technique is amenable to a high density multiwell format.

G. Improving pharmaceutical proteins using RSR

Table I lists proteins that are of particular commercial interest to the pharmaceutical industry. These proteins are all candidates for RSR evolution to improve function, such as specific activity, ligand binding, shelf life, reduction of side effects through enhanced specificity, etc. All are well-suited to manipulation by the techniques of the invention. Additional embodiments especially applicable to this list are described below.

First, high throughput methods for expressing and purifying libraries of mutant proteins, similar to the methods described above for Taq polymerase, are applied to the proteins of Table I. These mutants are screened for activity in a functional assay. For example, mutants of IL2 are screened for resistance to degradation by plasma or tissue proteases; or for retention of activity on the low affinity IL2 receptor but with loss of activity on the high affinity IL2 receptor. The genes from mutants with improved activity relative to wild-type are recovered, and subjected to RSR to improve the phenotype further.

Preferably, the libraries are generated in a display format such that the mature folded protein is physically linked to the genetic information that encodes it. Examples include phage display using filamentous phage (O'Neil et al., Current Biology 5:443-449

(1995)) or bacteriophage lambda gene V display (Dunn, J. Mol. Biol. 248:497-506 (1995)), peptides on plasmids (Gates et al., J. Mol. Biol. 255:373-386 (1995)) where the polypeptide of interest is fused to a lac headpiece dimer and the nascent translation product binds to a lac operator site encoded on the plasmid or PCR product, and polysome display (Mattheakis et al., Proc. Natl. Acad. Sci. (U.S.A.) 91:9022-9026 (1994)) where ribosomes are stalled on mRNA molecules such that the nascent polypeptide is exposed for interaction with cognate ligands without disrupting the stalled ribosome/mRNA complex. Selected complexes are subjected to RT-PCR to recover the genes.

When so displayed, affinity binding of the recombinant phage is often done using a receptor for the protein of interest. In some cases it is impractical to obtain purified receptor with retention of all desired biological characteristics (for example, 7-transmembrane (7-TM) receptors). In such cases, one could use cells expressing the receptor as the panning substrate. For example, Barry et al. (Nat. Med. 2:299-305 (1996)) have described successful panning of M13 libraries against whole cells to obtain phage that bind to the cells expressing a receptor of interest. This format could be generally applied to any of the proteins listed in Table I.

In some embodiments, the following method can be used for selection. A stock of phagemids encoding IFN alpha mutants, for example, can be used directly at suitable dilution to stimulate cells. The biological effect on the cells can be read out by standard assays (e.g., proliferation or viral resistance) or indirectly through the activation of a reporter gene such as GFP (Crameri et al., Nat. Med. 14:315-319 (1996)) under the control of an IFN responsive promoter, such as an MHC class I promoter. In one embodiment, phagemids remaining attached after stimulation, expression and FACS purification of the responsive cells, can be purified by FACS. Preferably, the brightest cells are collected. The phagemids are collected and their DNA subjected to RSR until the level of desired improvement is achieved.

Thus, for example, IL-3 is prepared in one of these display formats and subjected to RSR to evolve an agonist with a desired level of activity. A library of IL3 mutants on a filamentous phage vector is created and affinity selected ("panned") against purified IL3 receptor to obtain mutants with improved affinity for receptor or for improved potency of phase displayed IL-3. The mutant IL-3 genes are recovered by PCR, subjected to RSR, and recloned into the display vector. The cycle is repeated until the desired affinity or agonist activity is achieved.

Many proteins of interest are expressed as dimers or higher order multimeric forms. In some embodiments, the display formats descibed above preferentially are applied to a single chain version of the protein. Mutagenesis, such as RSR, can be used in these

display formats to evolve improved single chain derivatives of multimeric factors which initially have low but detectable activity. This strategy is described in more detail below.

H. Whole Cell Selections

In some embodiments, the eukaryotic cell is the unit of biological selection. The following general protocol can be used to apply RSR to the improvement of proteins using eukaryotic cells as the unit of selection: (1) transfection or transduction of libraries of mutants into a suitable host cell, (2) expression of the encoded gene product(s) either transiently or stably, (3) functional selection for cells with an improved phenotype (expression of a receptor with improved affinity for a target ligand; viral resistance, etc., (4) recovery of the mutant genes by, for example, PCR followed by preparation of HIRT supernatants with subsequent tranformation of *E. coli*, (5) RSR and (6) repetition of steps (1) - (5) until the desired degree of improvement is achieved.

For example, previous work has shown that one can use mammalian surface display to functionally select cells expressing cloned genes, such as using an antibody to clone the gene for an expressed surface protein (Reviewed by Seed, Curr. Opin. Biotechnol. 6:567-573 (1995)). Briefly, cells are transiently transfected with libraries of cloned genes residing on replicating episomal vectors. An antibody directed against the protein of interest (whose gene one wishes to clone) is immobilized on a solid surface such as a plastic dish, and the transfected cells expressing the protein of interest are affinity selected.

For example, the affinity of an antibody for a ligand can be improved using mammalian surface display and RSR. Antibodies with higher affinity for their cognate ligands are then screened for improvement of one or more of the following properties: (1) improved therapeutic properties (increased cell killing, neutralization of ligands, activation of signal transduction pathways by crosslinking receptors), (2) improved in vivo imaging applications (detection of the antibody by covalent/noncovalent binding of a radionuclide or any agent detectable outside of the body by noninvasive means, such as NMR), (3) improved analytical applications (ELISA detection of proteins or small molecules), and (4) improved catalysts (catalytic antibodies). The methods described are general and can be extended to any receptor-ligand pair of interest. A specific example is provided in the experimental section.

The use of a one mutant sequence-one transfected cell protocol is a preferred design feature for RSR based protocols because the point is to use functional selection to identify mutants with improved phenotypes and, if the transfection is not done in a "clonal" fashion, the functional phenotype of any given cell is the result of the sum of multiple transfected sequences. Protoplast fusion is one method to achieve this end, since each protoplast contains typically greater than 50 copies each of a single plasmid variant. However, it is a relatively low efficiency process (about 10^3 - 10^4 transfectants), and it does

not work well on some non-adherent cell lines such as B cell lines. Retroviral vectors provide a second alternative, but they are limited in the size of acceptable insert (<10 kb) and consistent, high expression levels are sometimes difficult to achieve. Random integration results in varying expression levels, thus introducing noise and limiting one's ability to distinguish between improvements in the affinity of the mutant protein vs. increased expression. A related class of strategies that can be used effectively to achieve "one gene-one cell" DNA transfer and consistent expression levels for RSR is to use a viral vector which contains a lox site and to introduce this into a host that expresses cre recombinase, preferably transiently, and contains one or more lox sites integrated into its genome, thus limiting the variability of integration sites (Rohlman et al. Nature Biotech. 14:1562-1565 (1996)).

An alternative strategy is to transfect with limiting concentrations of plasmid (i.e., about one copy per cell) using a vector that can replicate in the target cells, such as is the case with plasmids bearing SV40 origins transfected into COS cells. This strategy requires that either the host cell or the vector supply a replication factor such as SV40 large T antigen. Northrup et al. (J. Biol. Chem. 268:2917-2923 (1993)) describe a strategy wherein a stable transfectant expressing SV40 large T antigen is then transfected with vectors bearing SV40 origins. This format gave consistently higher transient expression and demonstrable plasmid replication, as assayed by sensitivity to digestion by Dpn I. Transient expression (i.e, non-integrating plasmids) is a preferred format for cellular display selections because it reduces the cycle time and increases the number of mutants that can be screened.

The expression of SV40 large T antigen or other replication factors may have deleterious effects on or may work inefficiently in some cells. In such cases, RSR is applied to the replication factor itself to evolve mutants with improved activity in the cell type of interest. A generic protocol for evolving such a factor is as follows:

The target cell is transfected with GFP cloned onto a vector containing SV40 large T antigen, an SV40 origin, and a reporter gene such as GFP; a related format is cotransfection with limiting amounts of the SV40 large T antigen expression vector and an excess of a reporter such as GFP cloned onto an SV40 origin containing plasmid. Typically after 1-10 days of transient expression, the brightest cells are purified by FACS. SV40 large T antigen mutants are recovered by PCR, and subjected to mutagenesis. The cycle is repeated until the desired level of improvement is obtained.

I. **Autocrine Selection**

In some embodiments, mutant proteins are selected or screened based on their ability to exert a biological effect in an autocrine fashion on the cell expressing the

mutant protein. For example, a library of alpha interferon genes can be selected for induction of more potent or more specific antiviral activity as follows. A library of interferon alpha mutants is generated in a vector which allows for induction of expression (i.e. under control of a metallothionein promoter) and efficient secretion in a multiwell format (96-well for example) with one or a few independent clones per well. In some embodiments, the promoter is not inducible,and may be constitutive.

Expression of the cloned interferon genes is induced. The cells are challenged with a cytotoxic virus against which one wishes to evolve an optimized interferon (for example vesicular stomatitus virus or HIV). Surviving cells are recovered. The cloned interferon genes are recovered by PCR amplification, subjected to RSR, and cloned back into the transfection vector and retransfected into the host cells. These steps are repeated until the desired level of antiviral activity is evolved.

In some embodiments, the virus of interest is not strongly cytotoxic. In this case a conditionally lethal gene, such as herpes simplex virus thymidine kinase, is cloned into the virus and after challenge with virus and recovery, conditionally lethal selective conditions are applied to kill cells that are infected with virus. An example of a conditionally lethal gene is herpes TK, which becomes lethal upon treating cells expressing this gene with the thymidine analog acyclovir. In some embodiments, the antiproliferative activity of the cloned interferons is selected by treating the cells with agents that kill dividing cells (for example, DNA alkylating agents).

In some embodiments, potent cytokines are selected by expressing and secreting a library of cytokines in cells that have GFP or another reporter under control of a promoter that is induced by the cytokine, such as the MHC class I promoter being induced by evolved variants of alpha interferon. The signal transduction pathway is configured such that the wild type cytokine to be evolved gives a weak but detectable signal.

J. **Improved Serum Stability and Circulation Half-Life**

In some embodiments of the invention, proteins are evolved by RSR to have improved circulation half life or stability in serum. A preferred method for improving half-life is evolving the affinity of a protein of interest for a long lived serum protein, such as an antibody or other abundant serum protein. Examples of how affinity for an antibody can enhance serum half life include the co-administration of IL2 and anti-IL2 antibodies which increases serum half-life and anti-tumor activity of human recombinant IL2 (Courtney et al., Immunopharmacology 28:223-232 (1994)).

The eight most abundant human serum proteins are serum albumin, immunoglobulins, lipoproteins, haptoglobin, fibrinogen, transferrin, alpha-1 antitrypsin, and alpha-2 macroglobulin (Doolittle, chapter 6, The Plasma Proteins F. Putnam, ed.; Academic

Press, 1984). These and other abundant serum proteins such as ceruloplasmin and fibronectin are the primary targets against which to evolve binding sites on therapeutic proteins such as in Table I for the purpose of extending half-life. In the case of antibodies, the preferred strategy is to evolve affinity for constant regions rather than variable regions in order to minimize individual variation in the concentration of the relevant target epitope (antibody V region usage between different individuals is significantly variable).

Binding sites of the desired affinity are evolved by applying phage display, peptides on plasmid display or polysome display selections to the protein of interest. As a source of diversity, one could randomly mutagenize an existing binding site or otherwise defined region of the target protein, append a peptide library to the N terminus, C terminus, or internally as a functionally nondisruptive loop, or use "family shuffling" of homologous genes. DNA shuffling is particularly advantageous for problems where one wants to simultaneously optimize two or more "uncorrelated" properties such as improved affinity for HSA while retaining biological activity.

In other embodiments of the invention, half life is improved by derivatization with PEG, other polymer conjugates or half-life extending chemical moieties. These are established methods for extending half-life of therapeutic proteins (R. Duncan, Clin. Pharmacokinet 27:290-306 (1994); Smith et al., TIBTECH 11 397-403 (1993)) and can have the added benefit of reducing immunogenicity (R. Duncan, Clin. Pharmacokinet 27:290-306 (1994)). However, derivatization can also result in reduced affinity of the therapeutic protein for its receptor or ligand. RSR is used to discover alternative sites in the primary sequence that can be substituted with lysine or other appropriate residues for chemical or enzymatic conjugation with half-life extending chemical moieties, and which result in proteins with maximal retention of biological activity.

A preferred strategy is to express a library of mutants of the protein in a display format, derivatize the library with the agent of interest (i.e. PEG) using chemistry that does not biologically inactivate the display system, select based on affinity for the cognate receptor, PCR amplify the genes encoding the selected mutants, shuffle, reassemble, reclone into the display format, and iterate until a mutant with the desired activity, post modification, is obtained. An alternative format is to express, purify and derivatize the mutants in a high throughput format, screen for mutants with optimized activity, recover the corresponding genes, subject the genes to RSR and repeat.

In further embodiments of the invention, binding sites for target human proteins that are localized in particular tissues of interest are evolved by RSR. For example, an interferon can be evolved to contain a binding site for a liver surface protein, such as hepatocyte growth factor receptor, such that the interferon partitions selectively onto liver cells and has higher specific antiviral activity on liver cells. Such an evolved interferon could

be useful for treatment of hepatitis. Analogously, one could evolve affinity for abundant epitopes on erythrocytes such as ABO blood antigens to localize a given protein to the blood stream.

In further embodiments of the invention, the protein of interest is evolved to have increased stability to proteases. For example, the clinical use of IL2 is limited by serious side effects that are related to the need to administer high doses. High doses are required due to the short half life (3-5 min, Lotze et al., JAMA 256(22):3117-3124 (1986)) and the consequent need for high doses to maintain a therapeutic level of IL2. One of the factors contributing to short half-lives of therapeutic proteins is proteolysis by serum proteases. Cathepsin D, a major renal acid protease, is responsible for the degradation of IL2 in Balb/c mice (Ohnishi et al., Cancer Res. 50:1107-1112 (1990)). Furthermore, Ohnishi showed that treatment of Balb/c mice with pepstatin, a potent inhibitor of this protease, prolongs the half life of recombinant human IL2 and augments lymphokine-activated killer cell activity in this mouse model.

Thus, evolution of protease resistant variants of IL2 or any of the proteins listed in Table I that are resistant to serum or kidney proteases is a preferred strategy for obtaining variants with extended serum half lives.

A preferred protocol is as follows. A library of the mutagenized protein of interest is expressed in a display system with a gene-distal epitope tag (i.e. on the N-terminus of a phage display construct such that if it is cleaved off by proteases, the epitope tag is lost). The expressed proteins are treated with defined proteases or with complex cocktails such as whole human serum. Affinity selection with an antibody to the gene distal tag is performed. A second screen or selection demanding biological function (e.g., binding to cognate receptor) is performed. Phage retaining the epitope tag (and hence protease resistant) are recovered and subjected to RSR. The process is repeated until the desired level of resistance is attained.

In other embodiments, the procedure is performed in a screening format wherein mutant proteins are expressed and purified in a high throughput format and screened for protease resistance with retention of biological activity.

In further embodiments of the invention, the protein of interest is evolved to have increased shelf life. A library of the mutagenized nucleic acid squence encoding the protein of interest is expressed in a display format or high throughput expression format, and exposed for various lengths of time to conditions for which one wants to evolve stability (heat, metal ions, nonphysiological pH of, for example, <6 or >8, lyophilization, freeze-thawing). Genes are recovered from functional survivors, for example, by PCR. The DNA is subjected to mutagenesis, such as RSR, and the process repeated until the desired level of improvement is achieved.

The case of IFN presents an opportunity to evolve recombinants with improved half-life. There are > 10^{26} possible recombinations of the amino acid diversity in this family. Since these recombinants are formed from segments of wild-type IFN genes, relatively few if any novel T cell epitopes will be created by the process. Molecules that are highly active are like to closely resemble natural interferons structurally, and thus present few if any novel B cell epitopes. This creates a situation wherein the ability to create large libraries of recombinants can be combined with the power of phage panning to select for recombinants with affinity for abundant serum proteins such as human serum albumin. Proteins with affinity for long lived, abundant serum proteins have been shown to have enhanced serum half lives. Thus, one could obtain IFN recombinants with lengthened serum half lives by using phage panning to select for recombinants which have affinity for proteins such as HSA. Since binding to HSA or the mutations which create affinity for HSA may abrogate or substantially reduce IFN activity, one would have to counter screen for retention of potent IFN activity. By applying phage panning, activity screening, and shuffling iteratively, one could obtain recombinants with high activity and a desired level of affinity for target serum proteins. The half lives of candidate IFN's can be tested in transgenic mice expressing the human serum protein as a neo-self protein.

These approaches can be generalized to other proteins for which there exist multiple homologous human allelic or nonallelic forms. The approach can also be generalized further to be applied to proteins with no non-allelic human homologs, such as IL2. The gene for IL2 would be shuffled with IL2 genes from other mammals, with a preference for closely related mammals such as the primates. Recombination of the "natural diversity" defined by these homologs is expected to generate very high quality libraries with many active and superior molecules as was seen for the activities of the shuffled interferons in human and mouse cells.

K. **Evolved Single Chain Versions of Multisubunit Factors**

As discussed above, in some embodiments of the invention, the substrate for evolution by RSR is preferably a single chain contruction. The possibility of performing asymetric mutagenesis on constructs of homomultimeric proteins provides important new pathways for further evolution of such constructs that is not open to the proteins in their natural homomultimeric states. In particular, a given mutation in a homomultimer will result in that change being present in each identical subunit. In single chain constructs, however, the domains can mutate independently of each other.

Conversion of multisubunit proteins to single chain constructs with new and useful properties has been demonstrated for a number of proteins. Most notably, antibody heavy and light chain variable domains have been linked into single chain Fv's (Bird et al.,

Science 242:423-426 (1988)), and this strategy has resulted in antibodies with improved thermal stability (Young et al., FEBS Lett 377:135-139 (1995)), or sensitivity to proteolysis (Solar et al., Prot. Eng. 8:717-723 (1995)). A functional single chain version of IL5, a homodimer, has been constructed, shown to have affinity for the IL5 receptor similar to that of wild type protein, and this construct has been used to perform assymetric mutagenesis of the dimer (Li et al., J. Biol. Chem. 271:1817-1820 (1996)). A single chain version of urokinase-type plasminogen activator has been made, and it has been shown that the single chain construct is more resistant to plasminogen activator inhibitor type 1 than the native homodimer (Higazi et al., Blood 87:3545-3549 (1996)). Finally, a single-chain insulin-like growth factor I/insulin hybrid has been constructed and shown to have higher affinity for chimeric insulin/IGF-1 receptors than that of either natural ligand (Kristensen et al., Biochem. J. 305:981-986 (1995)).

In general, a linker is constructed which joins the amino terminus of one subunit of a protein of interest to the carboxyl terminus of another subunit in the complex. These fusion proteins can consist of linked versions of homodimers, homomultimers, heterodimers or higher order heteromultimers. In the simplest case, one adds polypeptide linkers between the native termini to be joined. Two significant variations can be made. First, one can construct diverse libraries of variations of the wild type sequence in and around the junctions and in the linkers to facilitate the construction of active fusion proteins. Secondly, Zhang et al., (Biochemistry 32:12311-12318 (1993)) have described circular permutations of T4 lysozyme in which the native amino and carboxyl termini have been joined and novel amino and carboxyl termini have been engineered into the protein. The methods of circular permutation, libraries of linkers, and libraries of junctional sequences flanking the linkers allow one to construct libraries that are diverse in topological linkage strategies and in primary sequence. These libraries are expressed and selected for activity. Any of the above mentioned strategies for screening or selection can be used, with phage display being preferable in most cases. Genes encoding active fusion proteins are recovered, mutagenized, reselected, and subjected to standard RSR protocols to optimize their function. Preferably, a population of selected mutant single chain constructs is PCR amplified in two seprate PCR reactions such that each of the two domains is amplified separately. Oligonucieotides are derived from the 5' and 3' ends of the gene and from both strands of the linker. The separately amplified domains are shuffled in separate reactions, then the two populations are recombined using PCR reassembly to generate intact single chain constructs for further rounds of selection and evolution.

V. Improved Properties of Pharmaceutical Proteins

A. Evolved Specificity for Receptor or Cell Type of Interest

The majority of the proteins listed in Table I are either receptors or ligands of pharmaceutical interest. Many agonists such as chemokines or interleukins agonize more than one receptor. Evolved mutants with improved specificity may have reduced side effects due to their loss of activity on receptors which are implicated in a particular side effect profile. For most of these ligand/receptors, mutant forms with improved affinity would have improved pharmaceutical properties. For example, an antagonistic form of RANTES with improved affinity for CCR5 or CXCR4 or both should be an improved inhibitor of HIV infection by virtue of achieving greater receptor occupancy for a given dose of the drug. Using the selections and screens outlined above in combination with RSR, the affinities and specificities of any of the proteins listed in Table I can be improved. For example, the mammalian display format could be used to evolve TNF receptors with improved affinity for TNF.

Other examples include evolved interferon alpha variants that arrest tumor cell proliferation but do not stimulate NK cells, IL2 variants that stimulate the low affinity IL2 receptor complex but not the high affinity receptor (or vice versa), superantigens that stimulate only a subset of the V beta proteins recognized by the wild type protein (preferably a single V beta), antagonistic forms of chemokines that specifically antagonize only a receptor of interest, antibodies with reduced cross-reactivity, and chimeric factors that specifically activate a particular receptor complex. As an example of this latter case, one could make chimeras between IL2 and IL4, 7, 9, or 15 that also can bind the IL2 receptor alpha, beta and gamma chains (Theze et al., Imm. Today 17:481-486 (1996)), and select for chimeras that retain binding for the intermediate affinity IL2 receptor complex on monocytes but have reduced affinity for the high affinity IL2 alpha, beta, gamma receptor complex on activated T cells.

B. Evolved Agonists with Increased Potency

In some embodiments of the invention, a preferred strategy is the selection or screening for mutants with increased agonist activity using the whole cell formats described above, combined with RSR. For example, a library of mutants of IL3 is expressed in active form on phage or phagemids as described by Gram et al. (J. Immun. Meth. 161:169-176 (1993)). Clonal lysates resulting from infection with plaque-purified phage are prepared in a high through-put format such as a 96-well microtiter format. An IL3-dependent cell line expressing a reporter gene such as GFP is stimulated with the phage stocks in a high throughput 96-well. Phage that result in positive signals at the greatest dilution of phage supernatants are recovered; alternatively, DNA encoding the mutant IL3 can be recovered by PCR. In some embodiments, single cells expressing GFP under control of an IL3 responsive

promoter can be stimulated with the IL3 phagemid library, and the positive cells are FACS sorted. The recovered nucleic acid is then subjected to PCR, and the process repeated until the desired level of improvement is obtained.

Table I

POLYPEPTIDE CANDIDATES FOR EVOLUTION

Name	Name
Alpha-1 antitrypsin	Hedgehog proteins (e.g., Sonic, Indian, Desert)
Angiostatin	Hemoglobin (for blood substitute; for radiosensitization)
Antihemolytic factor	Hirudin
Apolipoprotein	Human serum albumin
Apoprotein	Insulin
Atrial natriuretic factor	Interferon gamma
Atrial natriuretic polypeptide	Interleukin 20 (melanoma differentiation associated gene 7)
Atrial peptides	Interleukins (1 to 18)
Bacillus thuringensis toxins (Bt toxins)	Lactoferrin
C chemikines (i.e., Lymphotactin)	Leptin
C-X-C chemokines (e.g., T39765, NAP-2, ENA-78, Gro-a, Gro-b, Gro-c, IP-10, GCP-2, NAP-4, SDF-1, PF4, MIG)	Leukemia inhibitiry factor (LIF)
Calcitonin	Luciferase
CC chemokines (e.g., Monocyte chemoattractant protein-1, Monocyte chemoattractant protein-2, Monocyte chemoattractant protein-3, Monocyte inflammatory protein-1 alpha, Monocyte inflammatory protein-1 beta, RANTES, I309, R83915, R91733, HCC1, T58847, D31065, T64262)	Neurturin
CD40 ligand	Neutrophil inhibitory factor (NIF)
Ciliary neurotrophic factor (CNTF)	Oncostatin-M
Collagen	Osteogenic protein
Colony stimulating factor (CSF, G-CSF, GM-CSF, M-CSF)	Parathyroid hormone
Complement factor 5a	Protein A
Complement inhibitor	Protein G
Complement receptor 1	RANK (receptor activator of NF-κβ)
Epidermal growth factor (EGF)	RANK ligand
Erythropoietin	Relaxin
Factor IX	Renin
Factor VII	Salmon calcitonin
Factor VIII	Salmon growth hormone
Factor X	Soluble CD4
Fibrinogen	Soluble CD28
Fibronectin	Soluble CD40
FLT-3 receptor antagonist	Soluble CD40 ligand
Glucocerebrosidase	Soluble CD80 (B7-1)
Gonadotropin	Soluble CD86 (B7-2)
Growth hormone	Soluble CD150 (SLAM)
	Soluble CD152 (CTLA-4)
	Soluble complement receptor I
	Soluble I-CAM 1
	Soluble INF gamma receptor
	Soluble interleukin receptors (IL-1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20)
	Soluble leptin receptor
	Soluble RANK
	Soluble TNF receptor

Somatomedin
Somatostatin
Somatotropin
Stem cell factor
Streptokinase
Superantigens, i.e., Staphylococcal enterotoxins (SEA, SEB, SEC1, SEC2, SEC3, SED, SEE), Toxic shock syndrome toxin (TSST-1), Exfoliating toxins A and B, Pyrogenic exotoxins A, B, and C, and M. arthritidis mitogen
Superoxide dismutase
Thrombopoietin
Thymosin alpha 1
Tissue plasminogen activator
Transforming growth factor beta
Tumor necrosis factor beta (TNF beta)
Tumor necrosis factor receptor (TNFR)
Tumor necrosis factor-alpha (TNF alpha)
Urokinase
Viral IL10 homologs

C. Evolution of Components of Eukaryotic Signal Transduction or Transcriptional Pathways

Using the screens and selections listed above, RSR can be used in several ways to modify eukaryotic signal transduction or transcriptional pathways. Any component of a signal transduction pathway of interest, or of the regulatory regions and transcriptional activators that interact with this region and with chemicals that induce transcription can be evolved. This generates regulatory systems in which transcription is activated more potently by the natural inducer or by analogues of the normal inducer. This technology is preferred for the development and optimization of diverse assays of biotechnological interest. For example, dozens of 7 transmembrane receptors (7-TM) are validated targets for drug discovery (see, for example, Siderovski et al., Curr Biol., 6(2):211-212 (1996); An et al., FEBS Lett., 375(1-2):121-124 (1995); Raport et al., Gene, 163(2):295-299 (1995); Song et al., Genomics, 28(2):347-349 (1995); Strader et al. FASEB J., 9(9):745-754 (1995); Benka et al., FEBS Lett., 363(1-2):49-52 (1995); Spiegel, J. Clin Endocrinol. Metab., 81(7):2434-2442 (1996); Post et al., FASEB J., 10(7):741-749 (1996); Reisine et al., Ann NY Acad. Sci., 780:168-175 (1996); Spiegel, Annu. Ref. Physiol., 58:143-170 (1996); Barak et al., Biochemistry, 34(47):15407-15414 (1995); and Shenker, Baillieres Clin. Endocrinol. Metab., 9(3):427-451 (1995)). The development of sensitive high throughput assays for agonists and antagonists of these receptors is essential for exploiting the full potential of combinatorial chemistry in discovering such ligands. Additionally, biodetectors or biosensors for different chemicals can be developed by evolving 7-TM's to respond agonistically to novel chemicals or proteins of interest. In this case, selection would be for contructs that are activated by the new chemical or polypeptide to be detected. Screening could be done simply with fluorescence or light activated cell sorting, since the desired improvement is coupled to light production.

In addition to detection of small molecules such as pharmaceutical drugs and environmental pollutants, biosensors can be developed that will respond to any chemical for

which there are receptors, or for which receptors can be evolved by recursive sequence recombination, such as hormones, growth factors, metals and drugs. The receptors may be intracellular and direct activators of transcription, or they may be membrane bound receptors that activate transcription of the signal indirectly, for example by a phosphorylation cascade. They may also not act on transcription at all, but may produce a signal by some post-transcriptional modification of a component of the signal generating pathway. These receptors may also be generated by fusing domains responsible for binding different ligands with different signalling domains. Again, recursive sequence recombination can be used to increase the amplitude of the signal generated to optimize expression and functioning of chimeric receptors, and to alter the specificity of the chemicals detected by the receptor.

For example, G proteins can be evolved to efficiently couple mammalian 7-TM receptors to yeast signal transduction pathways. There are 23 presently known G alpha protein loci in mammals which can be grouped by sequence and functional similarity into four groups, Gs (Gna, Gna1), Gi (Gnai-2, Gnai-3, Gnai-1, Gnao, Gnat-1, Gnat-2, Gnaz), Gq (Gnaq, Gna-11, Gna-14, Gna-15) and G12 (Gna-12, Gna-13) (B. Nurnberg et al., J Mol. Med., 73:123-132 (1995)). They possess an endogenous GTP-ase activity allowing reversible functional coupling between ligand-bound receptors and downstream effectors such as enzymes and ion channels. G alpha proteins are complexed noncovalently with G beta and G gamma proteins as well as to their cognate 7-TM receptor(s). Receptor and signal specificity are controlled by the particular combination of G alpha, G beta (of which there are five known loci) and G gamma (seven known loci) subunits. Activation of the heterotrimeric complex by ligand bound receptor results in dissociation of the complex into G alpha monomers and G beta, gamma dimers which then transmit signals by associating with downstream effector proteins. The G alpha subunit is believed to be the subunit that contacts the 7-TM, and thus it is a focal point for the evolution of chimeric or evolved G alpha subunits that can transmit signals from mammalian 7-TM's to yeast downstream genes.

Yeast based bioassays for mammalian receptors will greatly facilitate the discovery of novel ligands. Kang et al. (Mol. Cell Biol. 10:2582-2590 (1990)) have described the partial complementation of yeast strains bearing mutations in SCG1 (GPA1), a homologue of the alpha subunits of G proteins involved in signal transduction in mammalian cells, by mammalian and hybrid yeast/mammalian G alpha proteins. These hybrids have partial function, such as complementing the growth defect in scg1 strains, but do not allow mating and hence do not fully complement function in the pheromone signal transduction pathway. Price et al. (Mol. Cell Biol. 15:6188-6195 (1995)) have expressed rat somatostatin receptor subtype 2 (SSTR2) in yeast and demonstrated transmission of ligand binding signals by this 7-TM receptor through yeast and chimeric mammalian/yeast G alpha subunits

("coupling") to a HIS3 reporter gene, under control of the pheromone responsive promoter FUS-1 enabling otherwise HIS3(-) cells to grow on minimal medium lacking histidine.

Such strains are useful as reporter strains for mammalian receptors, but suffer from important limitations as exemplified by the study of Kang et al., where there appears to be a block in the transmission of signals from the yeast pheromone receptors to the mammalian G proteins. In general, to couple a mammalian 7-TM receptor to yeast signal transduction pathways one couples the mammalian receptor to yeast, mammalian, or chimeric G alpha proteins, and these will in turn productively interact with downstream components in the pathway to induce expression of a pheromone responsive promoter such as FUS-1. Such functional reconstitution is commonly referred to as "coupling".

The methods described herein can be used to evolve the coupling of mammalian 7-TM receptors to yeast signal transduction pathways. A typical approach is as follows: (1) clone a 7-TM of interest into a yeast strain with a modified pheromone response pathway similar to that described by Price (e.g., strains deficient in FAR1, a negative regulator of G_1 cyclins, and deficient in SST2 which causes the cells to be hypersensitive to the presence of pheromone), (2) construct libraries of chimeras between the mammalian G alpha protein(s) known or thought to interact with the GPA1 or homologous yeast G alpha proteins, (3) place a selectable reporter gene such as HIS3 under control of the pheromone responsive promoter FUS1 (Price et al., Mol. Cell Biol. 15:6188-6195 (1995)). Alternatively, a screenable gene such as luciferase may be placed under the control of the FUS1 promoter; (4) transform library (2) into strain (3) (HIS(-)), (5) screen or select for expression of the reporter in response to the ligand of interest, for example by growing the library of transformants on minimal plates in the presence of ligand to demand HIS3 expression, (6) recover the selected cells, and and apply RSR to evolve improved expression of the reporter under the control of the pheromone responsive promoter FUS1.

A second important consideration in evolving strains with optimized reporter constructs for signal transduction pathways of interest is optimizing the signal to noise ratio (the ratio of gene expression under inducing vs noninducing conditions). Many 7-TM pathways are leaky such that the maximal induction of a typical reporter gene is 5 to 10-fold over background. This range of signal to noise may be insufficient to detect small effects in many high through put assays. Therefore, it is of interest to couple the 7-TM pathway to a second nonlinear amplification system that is tuned to be below but near the threshold of activation in the uninduced state. An example of a nonlinear amplification system is expression of genes driven by the lambda P_L promoter. Complex cooperative interactions between lambda repressor bound at three adjacent sites in the cI promoter result in very efficient repression above a certain concentration of repressor. Below a critical threshold dramatic induction is seen and there is a window within which a small decrease in repressor

concentration leads to a large increase in gene expression (Ptashne, A Genetic Switch:Phage Lambda and Higher Organisms, Blackwell Scientific Publ. Cambridge, MA, 1992). Analogous effects are seen for some eukaryotic promoters such as those regulated by GAL4. Placing the expression of a limiting component of a transcription factor for such a promoter (GAL4) under the control of a GAL4 enhanced 7-TM responsive promoter results in small levels of induction of the 7-TM pathway signal being amplified to a much larger change in the expression of a reporter construct also under the control of a GAL4 dependent promoter.

An example of such a coupled system is to place GAL4 under control of the FUS-1 pheromone responsive promoter and to have the intracellular GAL4 (itself a transcriptional enhancer) level positively feedback on itself by placing a GAL4 binding site upstream of the FUS-1 promoter. A reporter gene is also put under the control of a GAL4 activated promoter. This system is designed so that GAL4 expression will nonlinearly self-amplify and co-amplify expression of a reporter gene such as luciferase upon reaching a certain threshold in the cell. RSR can be used to great advantage to evolve reporter constructs with the desired signaling properties, as follows: (1) A single plasmid construct is made which contains both the GAL4/pheromone pathway regulated GAL4 gene and the GAL4 regulated reporter gene. (2) This construct is mutagenized and transformed into the appropriately engineered yeast strain expressing a 7-TM and chimeric yeast/mammalian protein of interest. (3) Cells are stimulated with agonists and screened (or selected) based on the activity of the reporter gene. In a preferred format, luciferase is the reporter gene and activity is quantitated before and after stimulation with the agonist, thus allowing for a quantitative measurement of signal to noise for each colony. (4) Cells with improved reporter properties are recovered, the constructs are shuffled, and RSR is applied to further evolve the plasmid to give optimal signal to noise characteristics.

These approaches are general and illustrate how any component of a signal transduction pathway or transcription factor could be evolved using RSR and the screens and selections described above. For example, these specific methods could be used to evolve 7-TM receptors with specificity for novel ligands, specificity of nuclear receptors for novel ligands (for example to obtain herbicide or other small molecule-inducible expression of genes of interest in transgenic plants, such that a given set of genes can be induced upon treatment with a given chemical agent), specificity of transcription factors to be responsive to viral factors (thus inducing antiviral or lethal genes in cells expressing this transcription factor (transgenics or cells treated with gene therapy constructs), or specificity of transcription factors for activity in cancer cells (for example p53 deficient cells, thus allowing one to infect with gene therapy constructs expressing conditionally lethal genes in a tumor specific fashion).

The following examples are offered by way of illustration, not by way of limitation.

EXPERIMENTAL EXAMPLES

I. Evolution of BIAP

A preferred strategy to evolve BIAP is as follows. A codon usage libary is constructed from 60-mer oligonucleotides such that the central 20 bases of each oligo specifies the wild type protein, but encodes the wild-type protein sequence with degenerate codons. Preferably, very rare codons for the prokaryotic host of choice, such as *E. coli*, are not used. The 20 bases at each end of the oligo use non-degenerate, but preferred, codons in *E. coli*. The oligonucleotides are assembled into full-length genes as described above. The assembled products are cloned into an expression vector by techniques well known in the art. In some embodiments, the codon usage library is expressed with a library of secretory leader sequences, each of which directs the encoded BIAP protein to the *E. coli* periplasm. A library of leader sequences is used to optimize the combination of leader sequence and mutant. Examples of leader sequences are reviewed by Schatz et al. (Ann Rev. Genet. 24:215-248 (1990)). The cloned BIAP genes are expressed under the control of an inducible promoter such as the arabinose promoter. Arabinose- induced colonies are screened by spraying with a substrate for BIAP, bromo-chloro-indolyl phosphate (BCIP). The bluest colonies are picked visually and subjected to the RSR procedures described herein.

The oligonucleotides for construction of the codon usage library are listed in Table II. The corresponding locations of these promoters is provided in Figure 1.

Table II

1. AACCCTCCAG TTCCGAACCC CATATGATGA TCACCCTGCG TAAACTGCCG
2. AACCCTCCAG TTCCGAACCC CATATGAAAA AAACCGCT
3. AACCCTCCAG TTCCGAACCC ATATACATAT GCGTGCTAAA
4. AACCCTCCAG TTCCGAACCC CATATGAAAT ACCTGCTGCC GACC
5. AACCCTCCAG TTCCGAACCC GATATACATA TGAAACAGTC
6. TGGTGTTATG TCTGCTCAGG CDATGGCDGT DGAYTTYCAY CTGGTTCCGG TTGAAGAGGA
7. GGCTGGTTTC GCTACCGTTG CDCARGCDGC DCCDAARGAY CTGGTTCCGG TTGAAGAGGA
8. CACCCCGATC GCTATCTCTT CYTTYGCDTC YACYGGYTCY CTGGTTCCGG TTGAAGAGGA
9. GCTGCTGGCT GCTCAGCCGG CDATGGCDAT GGAYATYGGY CTGGTTCCGG TTGAAGAGGA

10. TGCCGCTGCT GTTCACCCCG GTDACYAARG CDGCDCARGT DCTGGTTCCG GTTGAAGAGG A

11. CCCGGCTTTC TGGAACCGTC ARGCDGCDCA RGCDCTGGAC GTTGCTAAAA AACTGCAGCC

12. ACGTTATCCT GTTCCTGGGT GAYGGYATGG GYGTDCCDAC CGTTACCGCT ACCCGTATCC

13. AAACTGGGTC CGGAAACCCC DCTGGCDATG GAYCARTTYC CGTACGTTGC TCTGTCTAAA

14. GGTTCCGGAC TCTGCTGGTA CYGCDACYGC DTAYCTGTGC GGTGTTAAAG GTAACTACCG

15. CTGCTCGTTA CAACCAGTGC AARACYACYC GYGGYAAYGA AGTTACCTCT GTTATGAACC

16. TCTGTTGGTG TTGTTACCAC YACYCGYGTD CARCAYGCDT CTCCGGCTGG TGCTTACGCT

17. GTACTCTGAC GCTGACCTGC CDGCDGAYGC DCARATGAAC GGTTGCCAGG ACATCGCTGC

18. ACATCGACGT TATCCTGGGT GGYGGYCGYA ARTAYATGTT CCCGGTTGGT ACCCCGGACC

19. TCTGTTAACG GTGTTCGTAA RCGYAARCAR AAYCTGGTDC AGGCTTGGCA GGCTAAACAC

20. GAACCGTACC GCTCTGCTGC ARGCDGCDGA YGAYTCYTCT GTTACCCACC TGATGGGTCT

21. AATACAACGT TCAGCAGGAC CAYACYAARG AYCCDACYCT GCAGGAAATG ACCGAAGTTG

22. AACCCGCGTG GTTTCTACCT GTTYGTDGAR GGYGGYCGYA TCGACCACGG TCACCACGAC

23. GACCGAAGCT GGTATGTTCG AYAAYGCDAT YGCDAARGCT AACGAACTGA CCTCTGAACT

24. CCGCTGACCA CTCTCACGTT TTYTCYTTYG GYGGYTAYAC CCTGCGTGGT ACCTCTATCT

25. GCTCTGGACT CTAAATCTTA YACYTCYATY CTGTAYGGYA ACGGTCCGGG TTACGCTCTG

26. CGTTAACGAC TCTACCTCTG ARGAYCCDTC YTAYCARCAG CAGGCTGCTG TTCCGCAGGC

27. AAGACGTTGC TGTTTTCGCT CGYGGYCCDC ARGCDCAYCT GGTTCACGGT GTTGAAGAAG

28. ATGGCTTTCG CTGGTTGCGT DGARCCDTAY ACYGAYTGYA ACCTGCCGGC TCCGACCACC

29. TGCTCACCTG GCTGCTTMAC CDCCDCCDCT GGCDCTGCTG GCTGGTGCTA TGCTGCTCCT C

30. TTCCGCCTCT AGAGAATTCT TARTACAGRG THGGHGCCAG GAGGAGCAGC ATAGCACCAG CC

31. AAGCAGCCAG GTGAGCAGCG TCHGGRATRG ARGTHGCGGT GGTCGGAGCC GGCAGGTT

32. CGCAACCAGC GAAAGCCATG ATRTGHGCHA CRAARGTYTC TTCTTCAACA CCGTGAACCA

33. GCGAAAACAG CAACGTCTTC RCCRCCRTGR GTYTCRGAHG CCTGCGGAAC AGCAGCCTGC

34. AGAGGTAGAG TCGTTAACGT CHGGRCGRGA RCCRCCRCCC AGAGCGTAAC CCGGACCGTT

35. AAGATTTAGA GTCCAGAGCT TTRGAHGGHG CCAGRCCRAA GATAGAGGTA CCACGCAGGG

36. ACGTGAGAGT GGTCAGCGGT HACCAGRATC AGRGTRTCCA GTTCAGAGGT CAGTTCGTTA

37. GAACATACCA GCTTCGGTCA GHGCCATRTA HGCYTTRTCG TCGTGGTGAC CGTGGTCGAT

38. GGTAGAAACC ACGCGGGTTA CGRGAHACHA CRCGCAGHGC AACTTCGGTC ATTTCCTGCA

39. TCCTGCTGAA CGTTGTATTT CATRTCHGCH GGYTCRAACA GACCCATCAG GTGGGTAACA

40. CAGCAGAGCG GTACGGTTCC AHACRTAYTG HGCRCCYTGG TGTTTAGCCT GCCAAGCCTG

41. TACGAACACC GTTAACAGAA GCRTCRTCHG GRTAYTCHGG GTCCGGGGTA CCAACCGGGA

42. CCCAGGATAA CGTCGATGTC CATRTTRTTH ACCAGYTGHG CAGCGATGTC CTGGCAACCG

43. CAGGTCAGCG TCAGAGTACC ARTTRCGRTT HACRGTRTGA GCGTAAGCAC CAGCCGGAGA

44. TGGTAACAAC ACCAACAGAT TTRCCHGCYT TYTTHGCRCG GTTCATAACA GAGGTAACTT

45. CACTGGTTGT AACGAGCAGC HGCRGAHACR CCRATRGTRC GGTAGTTACC TTTAACACCG

46. ACCAGCAGAG TCCGGAACCT GRCGRTCHAC RTTRTARGTT TTAGACAGAG CAACGTACGG

47. GGGTTTCCGG ACCCAGTTTA CCRTTCATYT GRCCYTTCAG GATACGGGTA GCGGTAACGG

48. CCCAGGAACA GGATAACGTT YTTHGCHGCR GTYTGRATHG GCTGCAGTTT TTTAGCAACG

49. ACGGTTCCAG AAAGCCGGGT CTTCCTCTTC AACCGGAACC AG

50. CCTGAGCAGA CATAACACCA GCHGCHACHG CHACHGCCAG CGGCAGTTTA CGCAGGGTGA

51. ACCGGGGTGA ACAGCAGCGG CAGCAGHGCC AGHGCRATRG TRGACTGTTT CATATGTATA TC

52. GCCGGCTGAG CAGCCAGCAG CAGCAGRCCH GCHGCHGCGG TCGGCAGCAG GTAGTTTCA

53. AAGAGATAGC GATCGGGGTG GTCAGHACRA TRCCCAGCAG TTTAGCACGC ATATGTATAT

54. CAACGGTAGC GAAACCAGCC AGHGCHACHG CRATHGCRAT AGCGGTTTTT TTCATATG

55 AGAATTCTCT AGAGGCGGAA ACTCTCCAAC TCCCAGGTT

56. TGAGAGGTTG AGGGTCCAAT TGGGAGGTCA AGGCTTGGG

All oligonucleotides listed 5' to 3'. The code for degenerate positions is: R: A or G; Y: C or T; H: A or C or T; D: A or G or T.

II. **Mammalian Surface Display**

During an immune response antibodies naturally undergo a process of affinity maturation resulting in mutant antibodies with improved affinities for their cognate antigens. This process is driven by somatic hypermutation of antibody genes coupled with clonal selection (Berek and Milstein, Immun. Rev. 96:23-41 (1987)). Patten et al. (Science 271:1086-1091 (1996)) have reconstructed the progression of a catalytic antibody from the germline sequence, which binds a p-nitrophenylphosphonate hapten with an affinity of 135 micromolar, to the affinity matured sequence which has acquired nine somatic mutations and binds with an affinity of 10 nanomolar. The affinity maturation of this antibody can be recapitulated and improved upon using cassette mutagenesis of the CDR's (or random mutagenesis such as with PCR), mammalian display, FACS selection for improved binding, and RSR to rapidly evolve improved affinity by recombining mutations encoding improved binding.

Genomic antibody expression shuttle vectors similar to those described by Gascoigne et al. (Proc. Natl. Acad. Sci. (U.S.A.) 84:2936-2940 (1987)) are constructed such that libraries of mutant V region exons can be readily cloned into the shuttle vectors. The kappa construct is cloned onto a plasmid encoding puromycin resistance and the heavy chain is cloned onto a neomycin resistance encoding vector. The cDNA derived variable region sequences encoding the mature and germline heavy and light chain V regions are reconfigured by PCR mutagenesis into genomic exons flanked by Sfi I sites with complementary Sfi I sites placed at the appropriate locations in the genomic shuttle vectors. The oligonucleotides used to create the intronic Sfi I sites flanking the VDJ exon are: 5' Sfi I: 5'-TTCCATTTCA TACATGGCCG AAGGGGCCGT GCCATGAGGA TTTT-3'; 3' Sfi I: 5'-TTCTAAATG CATGTTGGCC TCCTTGGCCG GATTCTGAGC CTTCAGGACC A-3'. Standard PCR mutagenesis protocols are applied to produce libraries of mutants wherein the following sets of residues (numbered according to Kabat, Sequences of Proteins of Immunological Interest, U.S. Dept of Health and Human Services, 1991) are randomized to NNK codons (GATC,GATC,GC):

Chain	CDR	Mutated residues
V-L	1	30, 31, 34
V-L	2	52, 53, 55
V-H	2	55, 56, 65
V-H	"4"	74, 76, 78

Stable transfectant lines are made for each of the two light and heavy chain constructs (mature and germline) using the B cell myeloma AG8-653 (a gift from J. Kearney) as a host using standard electroporation protocols. Libraries of mutant plasmids encoding the indicated libraries of V-L mutants are transfected into the stable transformant expressing the germline V-H; and the V-H mutants are transfected into the germline V-L stable transfectant line. In both cases, the libraries are introduced by protoplast fusion (Sambrook et al., Molecular Cloning, CSH Press (1987)) to ensure that the majority of transfected cells receive one and only one mutant plasmid sequence (which would not be the case for electroporation where the majority of the transfected cells would receive many plasmids, each expressing a different mutant sequence).

The p-nitrophenylphosphonate hapten (JWJ-1) recognized by this antibody is synthesized as described by Patten et al. (Science 271:1086-1091 (1996)). JWJ-1 is coupled directly to 5-(((2-aminoethyl)thio)acetyl)fluorescein (Molecular Probes, Inc.) by formation of an amide bond using a standard coupling chemistry such as EDAC (March, Advanced Organic Chemistry, Third edition, John Wiley and Sons, 1985) to give a monomeric JWJ-1-FITC probe. A "dimeric" conjugate (two molecules of JWJ-1 coupled to a FACS marker) is made in order to get a higher avidity probe, thus making low affinity

interactions (such as with the germline antibody) more readily detected by FACS. This is generated by staining with Texas Red conjugated to an anti-fluorescein antibody in the presence of two equivalents of JWJ-1-FITC. The bivalent structure of IgG then provides a homogeneous bivalent reagent. A spin column is used to remove excess JWJ-1-FITC molecules that are not bound to the anti-FITC reagent. A tetravalent reagent is made as follows. One equivalent of biotin is coupled with EDAC to two equivalents of ethylenediamine, and this is then be coupled to the free carboxylate on JWJ-1. The biotiylated JWJ-1 product is purified by ion exchange chromatography and characterized by mass spectrometry. FITC labelled avidin is incubated with the biotinylated JWJ-1 in order to generate a tetravalent probe.

The FACS selection is performed as follows, according to a protocol similar to that of Panka et al. (Proc. Natl. Acad. Sci. (U.S.A.) 85:3080-3084 (1988)). After transfection of libraries of mutant antibody genes by the method of protoplast fusion (with recovery for 36 - 72 hours), the cells are incubated on ice with fluorescently labelled hapten. The incubation is done on ice to minimize pinocytosis of the FITC conjugate which may contribute to nonspecific background. The cells are then sorted on the FACS either with or without a washing step. FACSing without a washing step is preferable because the off rate for the germline antibody prior to affinity maturation is expected to be very fast (>0.1 sec-1; Patten et al., Science 271:1086-1091 (1996)); a washing step adds a complicating variable. The brightest 0.1 - 10% of the cells are collected.

Four parameters are manipulated to optimize the selection for increased binding: monomeric vs dimeric vs tetrameric hapten, concentration of hapten used in the staining reaction (low concentration selects for high affinity Kd's), time between washing and FACS (longer time selects for low off rates), and selectivity in the gating (i.e. take the top 0.1% to 10%, more preferably the top 0.1%). The constructs expressing the germline, mature, and both combinations of half germline are used as controls to optimize this selectivity.

Plasmids are recovered from the FACS selected cells by the transformation of an *E. coli* host with Hirt supernatants. Alternatively, the mutant V gene exons are PCR-amplified from the FACS selected cells. The recovered V gene exons are subjected to RSR, recloned into the corresponding genomic shuttle vector, and the procedure recursively applied until the mean fluorescence intensity has increased. A relevant positive control for improved binding is transfection with the affinity matured 48G7 exons (Patten et al., op. cit.).

In a further experiment, equal numbers of germline and each of the two half germline transfectants are mixed. The brightest cells are selected under conditions described above. The V genes are recovered by PCR, recloned into expression vectors, and

co-transfected, either two plasmids per *E. coli* followed by protoplast fusion, or by bulk electroporation. The mean fluorescent intensity of the transfectants should increase due to enrichment of mature relative to germline V regions.

This methodology can be applied to evolve any receptor-ligand or binding partner interaction. Natural expression formats can be used to express libraries of mutants of any receptor for which one wants to improve the affinity for the natural or novel ligands. Typical examples would be improvement of the affinity of T cell receptors for ligands of interest (i.e. MHC/tumor peptide antigen complexes) or TNF receptor for TNF (soluble forms of TNF receptors are used therapeutically to neutralize TNF activity).

This format can also be used to select for mutant forms of ligands by expressing the ligand in a membrane bound form with an engineered membrane anchor by a strategy analogous to that of Wettstein et al.(J. Exp. Med. 174:219-28 (1991)). FACS selection is then performed with fluorescently labelled receptor. In this format one could, for example, evolve improved receptor antagonists from naturally occurring receptor antagonists (IL1 receptor antagonist, for example). Mutant forms of agonists with improved affinity for their cognate receptors could also be evolved in this format. These mutants would be candidates for improved agonists or potent receptor antagonists, analogous to reported antagonistic mutant forms of IL3.

III. **Evolution of Alpha Interferon**

There are at hand 18 known non-allelic human interferon-alpha (INF-α) genes, with highly related primary structures (78-95% identical) and with a broad range of biological activities. Many hybrid interferons with interesting biological activities differing from the parental molecules have been described (reviewed by Horisberger and Di Marco, Pharm. Ther. 66:507-534 (1995)). A consensus human alpha interferon, IFN-Con1, has been constructed synthetically wherein the most common residue in fourteen known IFN-α's has been put at each position, and it compares favorably with the naturally occurring interferons (Ozes et al., J. Interferon Res. 12:55-59 (1992)). This IFN contains 20 amino acid changes relative to IFN-α2a, the INF-α to which it is most closely related. IFN-Con1 has 10-fold higher specific antiviral activity than any known natural IFN subtype. IFN-α Con1 has in vitro activities 10 to 20 fold higher than that of recombinant IFN α-2a (the major IFN used clinically) in antiviral, antiproliferative and NK cell activation. Thus, there is considerable interest in producing interferon hybrids which combine the most desirable traits from two or more interferons. However, given the enormous number of potential hybrids and the lack of a crystal structure of IFN -α or of the IFN-α receptor, there is a perceived impasse in the development of novel hybrids (Horisberger and Di Marco, Pharm. Ther. 66:507-534 (1995)).

The biological effects of IFN-α's are diverse, and include such properties as induction of antiviral state (induction of factors that arrest translation and degrade mRNA); inhibition of cell growth; induction of Class I and Class II MHC; activation of monocytes and macrophages; activation of natural killer cells; activation of cytotoxic T cells; modulation of Ig synthesis in B cells; and pyrogenic activity.

The various IFN-α's subtypes have unique spectra of activities on different target cells and unique side effect profiles (Ortaldo et al., Proc. Natl. Acad. Sci. (U.S.A.) 81:4926-4929 (1984); Overall et al., J. Interferon Res. 12:281-288 (1992); Fish and Stebbing, Biochem. Biophys. Res. Comm. 112:537-546 (1983); Weck et al., J. Gen. Virol. 57:233-237 (1981)). For example, human IFNα has very mild side effects but low antiviral activity. Human IFNα8 has very high antiviral activity, but relatively severe side effects. Human IFNα7 lacks NK activity and blocks NK stimulation by other INFα's. Human IFN-α J lacks the ability to stimulate NK cells, but it can bind to the IFN-α receptor on NK cells and block the stimulatory activity of IFN-αA (Langer et al., J. Interferon Res. 6:97-105 (1986)).

The therapeutic applications of interferons are limited by diverse and severe side effect profiles which include flu-like symptoms, fatigue, neurological disorders including hallucination, fever, hepatic enzyme elevation, and leukopenia. The multiplicity of effects of IFN-α's has stimulated the hypothesis that there may be more than one receptor or a multicomponent receptor for the IFN-α family (R. Hu et al., J. Biol. Chem. 268:12591-12595 (1993)). Thus, the existence of abundant naturally occurring diversity within the human alpha IFN's (and hence a large sequence space of recombinants) along with the complexity of the IFN-α receptors and activities creates an opportunity for the construction of superior hybrids.

A. Complexity of the Sequence Space

Figure 2 shows the protein sequences of 11 human IFN-α's. The differences from consensus are indicated. Those positions where a degenerate codon can capture all of the diversity are indicated with an asterisk. Examination of the aligned sequences reveals that there are 57 positions with two, 15 positions with three, and 4 positions with four possible amino acids encoded in this group of alpha interferon genes. Thus, the potential diversity encoded by permutation of all of this naturally occurring diversity is: $2^{57} \times 3^{15} \times 4^4 = 5.3 \times 10^{26}$. Among these hybrids, of the 76 polymorphisms spread over a total of 175 sites in the 11 interferon genes, 171 of the 175 changes can be incorporated into homologue libraries using single degenerate codons at the corresponding positions. For example, Arg, Trp and Gly can all be encoded by the degenerate codon (A,T,G)GG. Using such a strategy, 1.3×10^{25} hybrids can be captured with a *single* set of degenerate oligonucleotides. As is evident from Tables III to VI, 27 oligonucleotides is sufficient to shuffle all eleven human

alpha interferons. Virtually all of the natural diversity is thereby encoded and fully permuted due to degeneracies in the nine "block" oligonucleotides in Table V.

B. Properties of a "Coarse Grain" Search of Homologue Sequence Space

The modelled structure of IFN alpha (Kontsek, Acta Vir. 38:345-360 (1994)) has been divided into nine segments based on a combination of criteria of maintaining secondary structure elements as single units and placing/choosing placement of the segment boundaries in regions of high identity. Hence, one can capture the whole family with a single set of mildly degenerate oligonucleotides. Table III and Figure 2 give the precise locations of these boundaries at the protein and DNA levels respectively. It should be emphasized that this particular segmentation scheme is arbitrary and that other segmentation schemes could also be pursued. The general strategy does not depend on placement of recombination boundaries at regions of high identity between the family members or on any particular algorithm for breaking the structure into segments.

Table III

Segmentation Scheme for Alpha Interferon

Segment	Amino Acids	# Alleles	# Permutations of all Sequence Variations
1	1-21	5	1024
2	22-51	10	6.2×10^4
3	52-67	6	96
4	68-80	7	1024
5	81-92	7	192
6	93-115	10	2.5×10^5
7	116-131	4	8
8	132-138	4	8
9	139-167	9	9216

Many of the IFN's are identical over some of the segments, and thus there are less than eleven different "alleles" of each segment. Thus, a library consisting of the permutations of the segment "alleles" would have a potential complexity of 2.1×10^7 (5 segment #1's times 10 segment #2's x .... x 9 segment # 9's). This is far more than can be examined in most of the screening procedures described, and thus this is a good problem for using RSR to search the sequence space.

C. Detailed Strategies for Using RSR to Search the IFN-alpha Homologue Sequence Space

The methods described herein for oligo directed shuffling (i.e. bridge oligonucleotides) are employed to construct libraries of interferon alpha hybrids, and the general methods described above are employed to screen or select these mutants for improved function. As there are numerous formats in which to screen or select for improved interferon activity, many of which depend on the unique properties of interferons, exemplary descriptions of IFN based assays are described below.

D. A Protocol for a Coarse Grain Search of Hybrid IFN Alpha Sequence Space

In brief, libraries are constructed wherein the 11 homologous forms of the nine segments are permuted (note that in many cases two homologues are identical over a given segment). All nine segments are PCR- amplified out of all eleven IFN alpha genes with the eighteen oligonucleotides listed in Table IV, and reassembled into full length genes with oligo directed recombination. An arbitrary number, e.g., 1000, clones from the library are prepared in a 96-well expression/purification format. Hybrids with the most potent antiviral activities are screened. Nucleic acid is recovered by PCR amplification, and subjected to recombination using bridge oligonucleotides. These steps are repeated until candidates with desired properties are obtained.

E. Strategies for Examining the Space of $>10^{26}$ Fine Grain Hybrids

In brief, each of the nine segments is synthesized with one degenerate oligo per segment. Degeneracies are chosen to capture all of the IFN-alpha diversity that can be captured with a single degenerate codon without adding any non-natural sequence. A second set of degenerate oligonucleotides encoding the nine segments is generated wherein all of the natural diversity is captured, but additional non-natural mutations are included at positions where necessitated by the constraints of the genetic code. In most cases all of the diversity can be captured with a single degenerate codon; in some cases a degenerate codon will capture all of the natural diversity but will add one non-natural mutation; at a few postions it is not possible to capture the natural diversity without putting in a highly degenerate codon which will create more than one non-natural mutation. It is at these positions that this second set of oligonucleotides will differ from the first set by being more inclusive. Each of the nine synthetic segments is then amplified by PCR with the 18 PCR oligonucleotides. Full length genes using the oligo directed recombination method are generated, transfected into a host, and assayed for hybrids with desired properties. The best hybrids from (e.g, the top 10%, 1% or 0.1%; preferably the top 1%) are subjected to RSR and the process repeated until a candidate with the desired properties is obtained.

F. "Non-gentle" Fine Grain Search

On the one hand, one could make libraries wherein each segment is derived from the degenerate synthetic oligonucleotides which will encode random permutations of the homologue diversity. In this case, the initial library will very sparsely search the space of $>10^{25}$ possible fine grain hybrids that are possible with this family of genes. One could proceed by breeding positives together from this search. However, there would be a large number of differences between independent members of such libraries, and consequently the breeding process would not be very "gentle" because pools of relatively divergent genes would be recombined at each step.

G. "Gentle" Fine Grain Search

One way to make this approach more "gentle" would be to obtain a candidate starting point and to gently search from there. This starting point could be either one of the natural IFN-alpha's (such as IFN alpha-2a which is the one that is being used most widely therapeutically), the characterized IFN-Con1 consensus interferon, or a hit from screening the shuffled IFN-alpha's described above. Given a starting point, one would make separate libraries wherein one breeds the degenerate segment libraries one at a time into the founder sequence. Improved hits from each library would then be bred together to gently build up mutations all throughout the molecule.

H. Functional Cellular Assays

The following assays, well known in the art, are used to screen IFN alpha mutants: inhibition of viral killing; standard error of 30-50%; inhibition of plaque forming units; very low standard error (can measure small effects); reduced viral yield (useful for nonlethal, nonplaque forming viruses); inhibition of cell growth (3H-thymidine uptake assay; activation of NK cells to kill tumor cells; suppression of tumor formation by human INF administered to nude mice engrafted with human tumors (skin tumors for example).

Most of these assays are amenable to high throughput screening. Libraries of recombinant IFN alpha mutants are expressed and purified in high throughput formats such as expression, lysis and purification in a 96-well format using anti-IFN antibodies or an epitope tag and affinity resin. The purified IFN preparations are screened in a high throughput format, scored, and the mutants encoding the highest activities of interest are subjected to further mutagenesis, such as RSR, and the process repeated until a desired level of activity is obtained.

I. Phage Display

Standard phage display formats are used to display biologically active IFN. Libraries of chimeric IFN genes are expressed in this format and are selected (positively or

negatively) for binding (or reduced binding) to one or more purified IFN receptor preparations or to one or more IFN receptor expressing cell types.

J. GFP or Luciferase Under Control of IFN-Alpha Dependent Promoter

Protein expressed by mutants can be screened in high throughput format on a reporter cell line which expresses GFP or luciferase under the control of an IFN alpha responsive promoter, such as an MHC Class I promoter driving GFP expression.

K. Stimulation of Target Cells with Intact Infections Particles

Purification of active IFN will limit the throughput of the assays described above. Expression of active IFN alpha on filamentous phage M13 would allow one to obtain homogenous preparations of IFN mutants in a format where thousands or tens of thousands of mutants could readily be handled. Gram et al. (J. Imm. Meth. 161:169-176 (1993)) have demonstrated that human IL3, a cytokine with a protein fold similar in topology to IFN alpha, can be expressed on the surface of M13 and that the resultant phage can present active IL3 to IL3 dependent cell lines. Similarly, Saggio et al. (Gene 152:35-39 (1995)) have shown that human ciliary neurotrophic factor, a four helix bundle cytokine, is biologically active when expressed on phage at concentrations similar to those of the soluble cytokine. Analogously, libraries of IFN alpha mutants on M13 can be expressed and phage stocks of defined titre used to present biologically active IFN in the high throughput assays and selections described herein.

The following calculation supports the feasibility of applying this technology to IFN alpha. Assuming (1) titres of $1x10^{10}$ phage/ml with five active copies of interferon displayed per phage, and (2) that the displayed interferon is equivalently active to soluble recombinant interferon (it may well be more potent due to multi-valency), the question then is whether one can reasonably expect to see biological activity.

($1x10^{10}$ phage/ml) x (5 IFN molecules/phage) x (1 mole/$6x10^{23}$ molecules) x ($26,000$ gm/mole) x (10^9 ng/gm) = 2.2 ng/ml

The range of concentration used in biological assays is: 1 ng/ml for NK activation, 0.1 - 10 ng/ml for antiproliferative activity on Eskol cells, and 0.1 - 1 ng/ml on Daudi cells (Ozes et al., J. Interferon Res. 12:55-59 (1992)). Although some subtypes are glycosylated, interferon alpha2a and consensus interferon are expressed in active recombinant form in *E. coli*, so at least these two do not require glycosylation for activity. Thus, IFN alpha expressed on filamentous phage is likely to be biologically active as phage stocks without further concentration. Libraries of IFN chimeras are expressed in phage display formats and scored in the assays described above and below to identify mutants with improved properties to be put into further rounds of RSR.

When one phage is sufficient to activate one cell due to the high valency state of the displayed protein (five per phage in the gene III format; hundreds per phage in the gene VIII format; tens in the lambda gene V format), then a phage stock can be used directly at suitable dilution to stimulate cells with a GFP reporter construct under the control of an IFN responsive promoter. Assuming that the phage remain attached after stimulation, expression and FACS purification of the responsive cells, one could then directly FACS purify hybrids with improved activity from very large libraries (up to and perhaps larger than 10^7 phage per FACS run).

A second way in which FACS is used to advantage in this format is the following. Cells can be stimulated in a multiwell format with one phage stock per well and a GFP type reporter construct. All stimulated cells are FACS purified to collect the brightest cells, and the IFN genes recovered and subjected to RSR, with iteration of the protocol until the desired level of improvement is obtained. In this protocol the stimulation is performed with individual concentrated lysates and hence the requirement that a single phage be sufficient to stimulate the cell is relaxed. Furthermore, one can gate to collect the brightest cells which, in turn, should have the most potent phage attached to them.

L. Cell Surface Display Protocol for IFN Alpha Mutants

A sample protocol follows for the cell surface display of IFN alpha mutants. This form of display has at least two advantages over phage display. First, the protein is displayed by a eukaryotic cell and hence can be expressed in a properly glycosylated form which may be necessary for some IFN alphas (and other growth factors). Secondly, it is a very high valency display format and is preferred in detecting activity from very weakly active mutants.

In brief, a library of mutant IFN's is constructed wherein a polypeptide signal for addition of a phosphoinositol tail has been fused to the carboxyl terminus, thus targeting the protein for surface expression (Wettstein et al., J. Exp. Med. 174:219-28 (1991)). The library is used to transfect reporter cells described above (luciferase reporter gene) in a microtiter format. Positives are detected with a charge coupling device(CCD) camera. Nucleic acids are recovered either by HIRT and retransformation of the host or by PCR, and are subjected to RSR for further evolution.

M. Autocrine Display Protocol for Viral Resistance

A sample protocol follows for the autocrine display of IFN alpha mutants. In brief, a library of IFN mutants is generated in a vector which allows for induction of expression (i.e. metallothionein promoter) and efficient secretion. The recipient cell line carrying an IFN responsive reporter cassette (GFP or luciferase) is induced by transfection

with the mutant IFN constructs. Mutants which stimulate the IFN responsive promoter are detected by by FACS or CCD camera.

A variation on this format is to challenge transfectants with virus and select for survivors. One could do multiple rounds of viral challenge and outgrowth on each set of transfectants prior to retrieving the genes. Multiple rounds of killing and outgrowth allow an exponential amplification of a small advantage and hence provide an advantage in detecting small improvements in viral killing.

Table IV

Oligonucleotides needed for blockwise recombination: 18

Oligonucleotides for alpha interferon shuffling

1. 5'-TGT[G/A]ATCTG[C/T]CT[C/G]AGACC
2. 5'-GGCACAAATG[G/A/C]G[A/C]AGAATCTCTC
3. 5'-AGAGATTCT[G/T]C[C/T/G]CATTTGTGCC
4. 5'-CAGTTCCAGAAG[A/G]CT[G/C][C/A]AGCCATC
5. 5'-GATGGCT[T/G][G/C]AG[T/C]CTTCTGGAACTG
6. 5'-CTTCAATCTCTTCA[G/C]CACA
7. 5'-TGTG[G/C]TGAAGAGATTGAAG
8. 5'-GGA[T/A][G/C]AGA[C/G][C/G]CTCCTAGA
9. 5'-TCTAGGAG[G/C][G/C]TCT[G/C][T/A]TCC
10. 5'-GAACTT[T/G/A][T/A]CCAGCAA[A/C]TGAAT
11. 5'-ATTCA[T/G]TTGCTGG[A/T][A/T/C]AAGTTC
12. 5'-GGACT[T/C]CATCCTGGCTGTG
13. 5'-CACAGCCAGGATG[G/A]AGTCC
14. 5'-AAGAATCACTCTTTATCT
15. 5'-AGATAAAGAGTGATTCTT
16. 5'-TGGGAGGTTGTCAGAGCAG
17. 5'-CTGCTCTGACAACCTCCCA
18. 5'-TCA[A/T]TCCTT[C/A]CTC[T/C]TTAA

Brackets indicate degeneracy with equal mixture of the specified bases at those positions. The purpose of the degeneracy is to allow this one set of primers to prime all members of the IFN family with similar efficiency. The choice of the oligo driven recombination points is important because they will get "overwritten" in each cycle of breeding and hence cannot coevolve with the rest of the sequence over many cycles of selection.

Table V

Oligonucleotides needed for "fine grain" recombination of natural diversity over each of the nine blocks

Block	#Length of oligo required
1	76
2	95
3	65
4	56
5	51
6	93
7	50
8	62
9	80

Table VI

Amino acids that can be reached by a single step mutation in the codon of interest.

Wild-Type Amino Acid	Amino acids reachable by one mutation
W	C, R, G, L
Y	F, S. C, H, N, D
F	L, I, V, S, Y, C
L	S, W, F, I, M, V, P
V	F, L, I, M, A, D, E, G
I	F, L, M, V, T, N, K, S, R
A	S, P, T, V, D, E, G
G	V, A, D, E, R, S, C, W
M	L, I, V, T, K, R
S	F, L, Y, C, W, P, T, A, R, G, N, T, I
T	S, P, A, I, M, N, K, S, R
P	S, T, A, L, H, Q, R
C	F, S, Y, R, G, W
N	Y, H, K, D, S, T, I
Q	Y, H, K, E, L, P, R
H	Y, Q, N, D, L, P, R
D	Y, H, N, E, V, A, G

E	Q, K, D, V, A, G
R	L, P, H, Q, C, W, S, G, K, T, I, M
K	Q, N, E, R, T, I, M

Based on this Table, the polymorphic positions in IFN alpha where all of the diversity can be captured by a degenerate codon have been identified. Oligonucleotides of the length indicated in Table V above with the degeneracies inferred from Table VI are synthesized.

N. **Evolution of Improved IFN-α**

1. **Cloning**

IFN genes were cloned by PCR amplification from genomic DNA with 12 sets of degenerate primers by methods as discussed generally above. The PCR products were cloned into a standard phagemid display vector as fusions to fd bacteriophage gene III. Thirty clones were sequenced and compared to human alpha IFN genes in the literature. Most of the sequence matched known sequences exactly or nearly exactly (>98%DNA identity). Several clones did not match well with any known IFNs (i.e., about 93% identity) and are candidate novel IFN genes. One gene was a clear recombinant which presumably was created during the PCR. Eight of the ten clones were pooled and shuffled. These eight sequences contain about 66% of the known amino acid changes in this gene family.

2. **Shuffling**

The genes were shuffled as follows. Pools of 20-50 bp and 50-100 bp fragments were prepared from partial Dnase I digests as described above. Additionally, 20-100 bp fragments were prepared from preparative PCR products of human genomic DNA with the same set of 12 primers. These fragments should contain all sequence diversity in the human alpha interferon locus. Chimeras were assembled by crossover PCR by 20 cycles of (94°C x 60", 6°C x 60", 25°C x 120") followed by two rounds of 1:10 dilution into PCR buffer and reassembly by 20 cycles of (94°C x 30", 40°C x 30", 72°C x (30+2n)") where n = cycle number. Full length genes were rescued by PCR with outside primers and the material was cloned into phagemid display vector by standard methods. Libraries of 2.5 x 10^4, 3.0 x 10^5 and 2 x 10^8 complexity were obtained from the 20-50 bp, 50-100 bp and genomic PCR fragments, respectively. Sequencing of random chimeras verified that the shuffling had worked efficiently.

3. **Validation of biological activity of phagemids**

Large scale preps of phagemid particles were made by standard methods, using M13 VCS as the helper phage. The IFN-geneIII fusion genes were induced at mid log phase by the addition of 0.02% arabinose. The PEG precipitated phagemid particles were

CsCl banded and dialyzed. The phagemid particles displayed active IFN as evidenced by the biological activity of phagemid preps expressing IFN-Con1, IFN2a, or the eight cloned wild type IFN's in a human Daudi cell antiproliferation assay (human cells) (Tymms et al., Genet. Anal. Techn. Appl. 7:53-63 (1990)).

4. Screening for improved activity in the Daudi assay

Two screening strategies were used to identify clones with improved activity: activity assays on randomly chosen clones and activity assays on CsCl banded pools followed by identification of the best clones from the most active pools.

As an example, among eight randomly chosen chimeras, three were more active than Con1, one was intermediate between Con1 and IFN2a, and four were negative. Figure 3 depicts the alignment of the amino acid sequences of four chimeric interferons with IFN-Con1.

An example of pooled clones follows. Ninety-six clones were combined into eight different pools of twelve and assayed as pools on Daudi cells. CsCl preps were made from the twelve clones in the most active pool (P12.7, or pool "F"). One of these clones, F4, was highly active with activity about 60x greater than Con1 and about 1000x greater than IFN2a. None of the parental IFN's had activity greater than Con1, so this represents an increase of about 60-fold relative to the best parental clone. This clone has been assayed in a human virus protection assay (WISH cells) (Jilbert et al., Microbial. Path. 1:159-168 (1986) and been found to be more active than Con1 in this assay as well, thus verifying bona fide interferon activity rather than generalized toxicity.

5. Evolution for activity on mouse cells

Eight wild-type mouse IFN genes were PCR amplified by standard methods and cloned into the phagemid vector. One of these clones was highly active in a mouse antiviral assay (mouse cells) (Beilharz et al., J. Interferon Res. 9:305-314 (1988) when displayed in this vector. The eight human parental IFN clones and IFN2a were all inactive; and Con1 was weakly active in the mouse antiviral assay. One of eight randomly screened human chimeras was more active than Con1. One of eight pools of 12 clones (Pool "G") was active in the mouse assay. Pool "G" yielded one highly active clone, G8. One of sixteen pools of ninety-six was active. This pool of ninety-six was broken into eight pools of twelve, and two of these pools were highly active.

6. Interpretation

Taken together, these data show that the recombination techniques described herein combined with the screening methods described herein can be used to improve the activity of already potent interferons on human cells. Additionally, the methods can be used to create a "related" activity (activity on mouse cells) that did not pre-exist at a detectable

level in the starting gene population. The data further demonstrate the applicability of the instant invention for creating populations of recombinant genes with Gaussian-like distributions of activities from which superior recombinants can be readily obtained.

IV. Evolution of an Improved Luciferase

The luciferase of *Photinus pyralis* was PCR amplified from pGL2_basic (Promega Corporation, Madison, WI). The luciferase of *Luciola mingrelica* was PCR amplified from pJGR (Devine et al., Biochim. Biophys. Acta 1173:121-132 (1993)). Both were cloned by their start codon, encoded by NcoI, into pBAD24 (Guzman et al., J. Bacter. 177:4121-4130 (1995)). For DNAseI digestion, the luciferase genes, including some flanking regions, were PCR amplified by the primers BADup (TGCACGGCGTCACACTTTGCTA) and BADdown (TACTGCCGCCAGGCAAATTCT). The PCR products were mixed in equimolar amounts and partially digested with DNAseI. Fragments from 70 to 280 bp were gel purified. Five µg fragments were assembled in a volume of 10 µl using Taq-polymerase and the following 15 cycles in a robocycler: 94°C, 30 seconds; 6°C, 60 seconds; 25°C, 180 seconds. The sample was diluted 1:6 and cycled for another 20 cycles using a 1:1 mix of Taq- and Pwo-polymerase in the DNA engine (94°C, 30 seconds; 40°C, 30 seconds, 72 °C, 30 seconds). The sample was diluted 1:4 and cycled for another 20 cycles using a 1:1 mix of Taq- and Pwo-polymerase in the DNA engine (94°C, 30 seconds; 40°C, 30 seconds; 72°, 30 seconds). To amplify the assembled DNA fragments, the assembly reaction was diluted 1:10 to 1:100 and the primers #773 (TAGCGGATCCTACCTGACGC) and #297 (TGAAAATCTTCTCTCATCCG) were included with the next 25 cycles using a 1:1 mix of Taq- and Pwo-polymerase in the DNA engine (94°C, 30 seconds; 45°C, 30 seconds; 72°C, 110 seconds). The PCR products were NcoI/HindIII digested and ligated into pCKX-GFP. pCKX-GFP is pBAD24, wherein the ClaI, NcoI Arabinose regulatory unit cassette was replaced by a variant of the *lux* autoinducer system of *Vibrio fischeri* from pJGR (Devine et al., Biochim. Biophys. Acta 1173:121-132 (1993)). The ligation was transformed into XL1-Blue. The libraries were plated on LB-Amp200 and grown ON at 37°C. The colonies were picked into six 384 well plates and grown overnight. The cultures were gridded onto nitrocellulose and the colonies were grown overnight (ON) at 30 °C. The plate was incubated for 45 min. at 60 °C. Then the nitrocellulose filter was placed onto a blotting paper containing 100 mM Na-citrate pH 5 containing 0.2% Triton X-100 and 1 mM D-Luciferin. This was placed onto plastic wrap with the nitrocellulose and colonies facing down. This assembly was placed on a BIOMAX MR in a film cassette for 30 min. After development, the film was scored by eye, the brightest clones were inoculated from the 384 well plates, and these clones were grown ON at 30°C in 75 µl LB-Amp in 96 well format. The luciferase was extracted from these cultures as follows. A culture volume of 20 µl was mixed with 20 µl

lysis buffer I (100 mM Tris-CL pH 7.8, 5% Triton X-100, 10 mM DTT, 10 mM EDTA, 2 mg/ml Polymyxin B sulfate). After shaking, the reaction mixture was frozen for 1 hr. at -70°C and thawed after that at room temperature. 60 µl of lysis buffer II (100 mM Tris-Cl pH 7.8, 0.25U/µl DNAseI, 1.5 mg/ml hen egg lysozyme, 40 mM MgSO4) were added and the lysis mixture was incubated for 30 min at room temperature. Aliquots of the lysates were incubated for 30 min at various temperatures between 30°C and 42°C. In addition, aliquots were left at RT for several days. The luciferase activity of 5 µl of the standard lysate and the heat treated lysates were measured using 50 µl complete assay buffer (20 mM Tris-Cl pH 7.8 5 mM MgSO4, 0.5 mM ATP, 0.5 mM Coenzyme A, 0.5 mM D-Luciferin, 5 mM DTT) in a Topcount luminometer. Several clones showed an increase in residual activity after heat treatment and when left at RT for several days. One clone showed an increase in luciferase activity of 5-fold over *Luciola mingrelica* wildtype clone in *E. coli* extracts when treated for 30 minutes at 39 °C. After 4 days incubation at RT, the same clone showed ten-fold more activity than wild-type *L. Mingrelica* luciferase that had been treated identically. In addition, this clone showed a significant increase (2-fold) in activity over wild-type when grown at 37°C.

These results demonstrate the evolution of a luciferase with improved stability relative to parental donor molecular substrates.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

All references cited herein are expressly incorporated in their entirety for all purposes.

WHAT IS CLAIMED IS:

1. A method for evolving a protein encoded by a DNA substrate molecule comprising:

(a) digesting at least a first and second DNA substrate molecule, wherein the at least a first and second substrate molecules differ from each other in at least one nucleotide, with a restriction endonuclease;

(b) ligating the mixture to generate a library of recombinant DNA molecules;

(c) screening or selecting the products of (b) for a desired property; and

(d) recovering a recombinant DNA substrate molecule encoding an evolved protein.

2. The method of claim 1, wherein the restriction endonuclease generates non-palindromic ends at cleavage sites.

3. The method of claim 1, wherein the substrate molecules have been engineered to contain at least one recognition site for a restriction endonuclease having non-palindromic ends at cleavage sites.

4. The method of claim 1, wherein (a) - (d) are repeated at least once.

5. The method of claim 1, wherein the DNA substrate molecule comprises a gene cluster.

6. The method of claim 1, wherein at least one restriction endonuclease fragment from a DNA substrate molecule is isolated and subjected to mutagenesis to generate a library of mutant fragments.

7. The method of step 6, wherein the library of mutant fragments is used in the ligation of (b).

8. The method of claim 7, wherein the DNA substrate molecule encodes all or part of a protein selected from Table I.

9. The method of claim 6, wherein mutagenesis comprises recursive sequence recombination.

10. The method of claim 1, wherein the products of (d) are subjected to mutagenesis.

11. The method of claim 10, wherein mutagenesis comprises recursive sequence recombination.

12. The method of claim 1, wherein the products of (d) are used as a DNA substrate molecule in (b).

13. The method of claim 10, wherein the products of claim 10 are used in (d).

14. The method of claim 1, wherein the recombinant DNA substrate molecule of (d) comprises a library of recombinant DNA substrate molecules.

15. An evolved protein produced by the method of claim 1.

16. A method for evolving a protein encoded by a DNA substrate molecule by recombining at least a first and second DNA substrate molecule, wherein the at least a first and second substrate molecules differ from each other in at least one nucleotide and comprise defined segments, the method comprising:

(a) providing a set of oligonucleotide PCR primers, comprising at least one primer for each strand of each segment, wherein the primer sequence is complementary to at least one junction with another segment;

(b) amplifying the segments of the at least a first and second DNA substrate molecules with the primers of step (a) in a polymerase chain reaction;

(c) assembling the products of step (b) to generate a library of recombinant DNA substrate molecules;

(d) screening or selecting the products of (c) for a desired property; and

(e) recovering a recombinant DNA substrate molecule from (d) encoding an evolved protein.

17. The method of claim 16, wherein the at least a first and second DNA substrate molecules are subjected to mutagenesis prior to step (a).

18. The method of claim 16, wherein the at least a first and second DNA substrate molecules comprise alleles of a gene.

19. The method of claim 16, wherein the at least a first and second DNA substrate molecules comprise a library of mutants.

20. The method of claim 16, wherein the segments are defined by sites within intergenic regions.

21. The method of claim 16, wherein the segments are defined by sites within introns.

22. The method of claim 16, wherein the primers comprise a uracil substitution at one or more thymidine residues.

23. The method of claim 22, wherein the products of (b) are treated with uracil glycosylase.

24. The method of claim 16, wherein (a) - (e) are repeated at least once.

25. The method of claim 16, wherein the at least a first and second DNA substrate molecule comprises a gene cluster.

26. The method of claim 16, wherein the at least first and second DNA substrate molecule encodes all or part of a DNA polymerase.

27. The method of claim 16, wherein at least one PCR primer differs from the at least a first and second DNA substrate molecules in at least one nucleotide.

28. The method of claim 27, wherein the PCR primer comprises a nucleotide sequence of a known mutant or polymorphism of the at least a first or second DNA substrate molecule.

29. The method of claim 28, wherein the PCR primer comprises a set of degenerate primers which encode the nucleotide sequences of more than one known mutant or polymorphism of the at least a first or second DNA substrate molecule.

30. The method of claim 29, wherein the at least a first and second DNA substrate molecule encodes all or part of a protein selected from Table I.

31. The method of claim 17, wherein mutagenesis comprises recursive sequence recombination.

32. The method of claim 16, wherein the products of (e) are subjected to mutagenesis.

33. The method of claim 32, wherein mutagenesis comprises recursive sequence recombination.

34. The method of claim 32, wherein the products of claim 32 are used in (b).

35. The method of claim 16, wherein the products of (e) are used as a DNA substrate molecule in (b).

36. The method of claim 16, wherein the recombinant DNA substrate molecule of (e) comprises a library of recombinant DNA substrate molecules.

37. An evolved protein produced by the method of claim 16.

38. A method of enriching a population of DNA fragments for mutant sequences comprising:

(a) denaturing and renaturing the population of fragments to generate a population of hybrid double-stranded fragments in which at least one double-stranded fragment comprises at least one base pair mismatch;

(b) fragmenting the products of (a) into fragments of about 20-100 bp;

(c) affinity-purifying fragments having a mismatch on an affinity matrix to generate a pool of DNA fragments enriched for mutant sequences; and

(d) assembling the products of (c) to generate a library of recombinant DNA substrate molecules.

39. The method of claim 38, wherein the population of DNA fragments is derived from at least a first and second DNA substrate molecule, the at least a first and second DNA substrate molecule differing from each other in at least one nucleotide.

40. The method of claim 39, wherein the at least a first and second DNA substrate molecules are obtained by mutagenesis of a DNA substrate molecule.

41. The method of claim 39, wherein the at least a first and second DNA substrate molecules comprises alleles of a gene.

42. The method of claim 39, wherein the at least a first and second DNA substrate molecules comprises polymorphic variants of a gene.

43. The method of claim 38, wherein the DNA substrate molecule encodes all or part of a protein selected from Table I.

44. The method of claim 38, wherein the products of (c) are mixed with the products of (a) prior to (d).

45. A method for evolving a protein encoded by a DNA substrate molecule, the method comprising recombining at least a first and second DNA substrate molecule, wherein the at least a first and second substrate molecules share a region of sequence homology of about 10 to 100 base pairs and comprise defined segments, the method comprising:

(a) providing regions of homology in the at least a first and second DNA substrate molecules by inserting an intron sequence between at least two defined segments;

(b) fragmenting and recombining DNA substrate molecules of (a), wherein regions of homology are provided by the introns;

(c) screening or selecting the products of (b) for a desired property; and

(d) recovering a recombinant DNA substrate molecule from the products of (c) encoding an evolved protein.

46. The method of claim 45, wherein the introns are self-splicing.

47. The method of claim 45, wherein the inserted introns comprise from about 1 to about 10 nonhomologous introns.

48. The method of claim 45, wherein the intron comprises a recognition site for a restriction endonucleases having non-palindromic ends at cleavage sites.

49. The method of claim 45. wherein (b) - (d) are repeated at least once.

50. The method of claim 45, wherein the DNA substrate molecule comprises a gene cluster.

51. The method of claim 45, wherein at least one segment from a DNA substrate molecule is isolated and subjected to mutagenesis to generate a library of mutant fragments.

52. The method of claim 51, wherein the library of mutant segments is used in the recombination of (b).

53. The method of claim 45, wherein the segments are defined by exons.

54. The method of claim 45, wherein the segments are defined by intergenic regions.

55. The method of claim 45, wherein the at least a first and second DNA substrate molecules encode protein homologues.

56. The method of claim 45, wherein the intron contains a lox site, and wherein the products of (b) are used to transfect a Cre^+ host.

57. The method of claim 45, wherein the at least a first and second DNA substrate molecule encodes all or part of a protein selected from Table I.

58. The method of claim 45, wherein the at least a first and second DNA substrate molecule are subjected to mutagenesis prior to step (a).

59. The method of claim 58, wherein the mutagenesis comprises recursive sequence recombination.

60. The method of claim 45, wherein the products of (d) are subjected to mutagenesis.

61. The method of claim 58, wherein the mutagenesis comprises recursive sequence recombination.

62. The method of claim 45, wherein the products of (d) are used as a DNA substrate molecule in (b).

63. The method of claim 45, wherein the recombinant DNA substrate molecule of (d) comprises a library of recombinant DNA substrate molecules.

64. An evolved protein produced by the method of claim 45.

65. A method for evolving a protein encoded by a DNA substrate molecule by recombining at least a first and second DNA substrate molecule, wherein the at least a first and second substrate molecules differ from each other in at least one nucleotide and comprise defined segments, the method comprising:

(a) providing a set of oligonucleotide PCR primers, wherein for each junction of segments a pair of primers is provided, one member of each pair bridging the junction at one end of a segment and the other bridging the junction at the other end of the segment, with the terminal ends of the DNA molecule having as one member of the pair a generic primer, and wherein a set of primers is provided for each of the at least a first and second substrate molecules;

(b) amplifying the segments of the at least a first and second DNA substrate molecules with the primers of (a) in a polymerase chain reaction;

(c) assembling the products of (b) to generate a pool of recombinant DNA molecules;

(d) selecting or screening the products of (c) for a desired property; and

(e) recovering a recombinant DNA substrate molecule from the products of (d) encoding an evolved protein.

66. The method of claim 65, wherein (a) - (e) is repeated at least once.

67. The method of claim 65, wherein the at least a first and second DNA substrate molecule are subjected to mutagenesis prior to (a).

68. The method of claim 65, wherein the at least a first and second DNA substrate molecule comprise sequences encoding protein homologues.

69. The method of claim 65, wherein the primers comprise a uracil substitution at one or more thymidine residues.

70. The method of claim 69, wherein the products of (b) are treated with uracil glycosylase.

71. The method of claim 65, wherein the at least a first and second DNA substrate molecule encodes all or part of a protein selected from Table I.

72. The method of claim 65, wherein the at least a first and second DNA substrate molecule comprises a gene cluster.

73. An evolved protein produced by the method of claim 65.

74. The method of claim 65, wherein at least one PCR primer differs from the at least a first and second substrate molecules in at least one nucleotide.

75. The method of claim 74, wherein the PCR primer comprises a nucleotide sequence of a known mutant or polymorphism of the at least a first or second substrate molecule.

76. The method of claim 75, wherein the PCR primer is degenerate and encodes the nucleotide sequences of more than one known mutant or polymorphism of the at least a first or second substrate molecule.

77. The method of claim 67, wherein mutagenesis comprises recursive sequence recombination.

78. The method of claim 65, wherein the products of (e) are subjected to mutagenesis.

79. The method of claim 78, wherein mutagenesis comprises recursive sequence recombination.

80. The method of claim 65, wherein the products of (e) are used as a DNA substrate molecule in (b).

81. The method of claim 65, wherein the recombinant DNA substrate molecule of (e) comprises a library of recombinant DNA substrate molecules.

82. A method for optimizing expression of a protein by evolving the protein, wherein the protein is encoded by a DNA substrate molecule, comprising:

(a) providing a set of oligonucleotides, wherein each oligonucleotide comprises at least two regions complementary to the DNA molecule and at least one degenerate region, each degenerate region encoding a region of an amino acid sequence of the protein;

(b) assembling the set of oligonucleotides into a library of full length genes;

(c) expressing the products of (b) in a host cell;

(d) screening the products of (c) for improved expression of the protein; and

(e) recovering a recombinant DNA substrate molecule encoding an evolved protein from (d).

83. The method of claim 82, wherein the primers comprise about 20 nucleotides complementary to the DNA substrate molecule followed by a second region of about 20 degenerate nucleotides of homology with the DNA substrate molecules followed by about 20 nucleotides complementary to the DNA substrate.

84. The method of claim 82, wherein the protein is bovine intestinal alkaline phosphatase.

85. The method of claim 84, wherein the oligonucleotides comprise one or more primers from Table II.

86. The method of claim 82, wherein the DNA substrate molecule encodes all or part of a protein selected from Table I.

87. The method of claim 82, wherein the DNA molecule comprises a gene cluster.

88. The method of claim 82, wherein (a) - (e) are repeated at least once.

89. The method of claim 82, wherein the oligonucleotides comprise at least 5' and 3' nucleotide complementary to the DNA substrate molecule and about 20-300 nucleotides having up to about 85% sequence homology with a region of the DNA substrate molecule.

90. The method of claim 89, wherein the oligonucleotides comprise a set of oligonucleotides in which each oligonucleotide overlaps with a second oligonucleotide.

91. The method of claim 82, wherein the products of (e) are subjected to mutagenesis.

92. The method of claim 91, wherein mutagenesis comprises recursive sequence recombination.

93. The method of claim 82, wherein the recombinant DNA substrate molecule of (e) comprises a library of recombinant DNA substrate molecules.

94. An evolved protein produced by the method of claim 82.

95. A method for optimizing expression of a protein encoded by a DNA substrate molecule by evolving the protein, wherein the DNA substrate molecule comprises at least one lac operator and a fusion of a DNA sequence encoding the protein with a DNA sequence encoding a lac headpiece dimer, the method comprising:

(a) transforming a host cell with a library of mutagenized DNA substrate molecules;

(b) inducing expression of the protein encoded by the library of (a);

(c) preparing an extract of the product of (b);

(d) fractionating insoluble protein from complexes of soluble protein and DNA; and

(e) recovering a DNA substrate molecule encoding an evolved protein from (d).

96. The method of claim 95, wherein (a) - (e) are repeated at least once.

97. The method of claim 95, wherein the DNA substrate molecule encodes all or part of a protein selected from Table I.

98. An evolved protein produced by the method of claim 95.

99. The method of claim 95, wherein the products of (e) are subjected to mutagenesis.

100. The method of claim 99, wherein mutagenesis comprises recursive sequence recombination.

101. The method of claim 95, wherein the products of (e) are used as a DNA substrate molecule in (a).

102. The method of claim 95, wherein the recombinant DNA substrate molecule of (e) comprises a library of recombinant DNA substrate molecules.

103. A method for evolving functional expression of a protein encoded by a DNA substrate molecule comprising a fusion of a DNA sequence encoding the protein with a DNA sequence encoding filamentous phage protein to generate a fusion protein, the method comprising:

(a) providing a host cell producing infectious particles expressing a fusion protein encoded by a library of mutagenized DNA substrate molecules;

(b) recovering from (a) infectious particles displaying the fusion protein;

(c) affinity purifying particles displaying the mutant protein using a ligand for the protein; and

(d) recovering a DNA substrate molecule encoding an evolved protein from affinity purified particles of (c).

104. The method of claim 103, wherein (a) - (d) are repeated at least once.

105. The method of claim 103, wherein the DNA substrate molecule encodes all or part of a protein selected from Table I.

106. An evolved protein produced by the method of claim 103.

107. The method of claim 103, wherein the products of (d) are subjected to mutagenesis.

108. The method of claim 107, wherein mutagenesis comprises recursive sequence recombination.

109. The method of claim 107, wherein the products of claim 107 are used as a DNA substrate molecule in (a).

110. The method of claim 103, wherein the DNA substrate molecule of (e) comprises a library of DNA substrate molecules.

111. The method of claim 103, wherein DNA sequence encoding the filamentous phage protein comprises a phagemid.

112. The method of claim 103, wherein DNA sequence encoding the filamentous phage protein comprises a phage.

113. A method for optimizing expression of a protein encoded by a DNA substrate molecule comprising a fusion of a DNA sequence encoding the protein with a DNA substrate encoding a lac headpiece dimer, wherein the DNA substrate molecule is present on a first plasmid vector, the method comprising:

(a) providing a host cell transformed with the first vector and a second vector comprising a library of mutants of at least one chaperonin geneand at least one lac operator;

(b) preparing an extract of the product of (a);

(c) fractionating insoluble protein from complexes of soluble protein and DNA; and

(d) recovering DNA encoding a chaperonin gene from (c).

114. The method of claim 113, wherein the DNA substrate molecule encodes all or part of a protein selected from Table I.

115. The method of claim 113, wherein the DNA substrate is subjected to mutagenesis independently of the chaperonin gene prior to (a).

116. The method of claim 113, wherein the DNA of (d) comprises a library of mutants.

117. The method of claim 113, wherein the first and second vectors are the same vector.

118. The method of claim 113, wherein (d) further comprises recovering an evolved DNA substrate molecule from the products of (c).

119. An evolved chaperonin produced by the method of claim 113.

120. An evolved protein produced by the method of claim 113.

121. The method of claim 113, wherein (a) - (d) are repeated at least once.

122. The method of claim 113, wherein the products of (d) are subjected to mutagenesis.

123. The method of claim 122, wherein mutagenesis comprises recursive sequence recombination.

124. The method of claim 122, wherein the products of claim 122 are used in (a).

125. A method for optimizing expression of a protein encoded by a DNA substrate molecule comprising a fusion of a DNA sequence encoding the protein with a filamentous phage gene, wherein the fusion is carried on a phagemid comprising a library of chaperonin gene mutants, the method comprising:

(a) providing a host cell producing infectious particles expressing a fusion protein encoded by a library of mutagenized DNA substrate molecules;

(b) recovering from (a) infectious particles displaying the fusion protein;

(c) affinity purifying particles displaying the protein using a ligand for the protein; and

(d) recovering DNA encoding the mutant chaperonin from affinity purified particles of (c).

126. The method of claim 125, wherein (a) - (d) are repeated at least once.

127. The method of claim 125, wherein the DNA substrate molecule encodes all or part of a protein selected from Table I.

128. An evolved chaperonin produced by the method of claim 125.

129. An evolved protein produced by the method of claim 125.

130. The method of claim 125, wherein the products of (d) are subjected to mutagenesis.

131. The method of claim 130, wherein mutagenesis comprises recursive sequence recombination.

132. The method of claim 130, wherein the products of claim 130 are used in (a).

133. The method of claim 125, wherein the DNA of (d) comprises a library of DNA substrate molecules.

134. The method of claim 125, wherein the DNA substrate molecule comprises a library of mutagenized DNA sequences encoding the protein of interest.

135. The method of claim 125, wherein (d) further comprises recovering DNA encoding the protein from affinity purified particles of (c).

136. A method for optimizing secretion of a protein in a host by evolving a gene encoding a secretory function, comprising:

(a) providing a cluster of genes encoding secretory functions;

(b) recombining at least a first and second sequence in the gene cluster of (a) encoding a secretory function, the at least a first and second sequences differing from each other in at least one nucleotide, to generate a library of recombinant sequences;

(c) transforming a host cell culture with the products of (b), wherein the host cell comprises a DNA sequence encoding the protein;

(d) subjecting the product of (c) to screening or selection for secretion of the protein; and

(e) recovering DNA encoding an evolved gene encoding a secretory function from the product of (d).

137. The method of claim 136, wherein the gene cluster comprises at least one recognition site for a restriction endonuclease having nonpalindromic ends at the cleavage site.

138. The method of claim 136, wherein the host is *E. coli.*, yeast, *Bacillus*, *Pseudomonas*, or a mammalian cell.

139. The method of claim 136, wherein the protein is a thermostable DNA polymerase.

140. The method of claim 136, wherein protein is inducibly expressed.

141. The method of claim 136, wherein the protein is linked to a secretory leader sequence.

142 A secretory gene evolved by the method of claim 136.

143. The method of claim 136, wherein (a) - (e) are repeated at least once.

144 The method of claim 136, wherein the DNA sequence of (c) encodes all or part of a protein selected from Table I.

145 The method of claim 136, wherein the DNA sequence of (c) comprises a library of mutant sequences.

146. The method of claim 136, wherein the products of (e) are subjected to mutagenesis.

147 The method of claim 146, wherein mutagenesis comprises recursive sequence recombination.

148 The method of claim 146, wherein the products of claim 146 are used in (a).

149. The method of claim 136, wherein the DNA of (e) comprises a library of evolved genes.

150. A method for evolving an improved DNA polymerase comprising:

(a) providing a library of mutant DNA substrate molecules encoding mutant DNA polymerase;

(b) screening extracts of cells transfected with (a) and comparing activity with wild type DNA polymerase;

(c) recovering mutant DNA substrate molecules from cells in (b) expressing mutant DNA polymerase having improved activity over wild-type DNA polymerase; and

(d) recovering a DNA substrate molecule encoding an evolved polymerase from the products of (c).

151. The method of claim 150, wherein the improved activity is at least one of the group of higher quality sequencing ladder. less termination of reactions with inosine, improve acceptance of base analogs, improved acceptance of dideoxy nucleotides, and longer sequencing ladders.

152. The method of claim 150, wherein the products of (a) are expressed under control of arabinose promoter in an E. coli host having a mutant host DNA polymerase.

153. The method of claim 150, wherein (a) - (d) are repeated at least once.

154. An evolved DNA polymerase produced by the method of claim 150.

155. The method of claim 150, wherein the products of (d) are subjected to mutagenesis.

156. The method of claim 155, wherein mutagenesis comprises recursive sequence recombination.

157. The method of claim 155, wherein the products of claim 155 are used in (a).

158. The method of claim 150, wherein the DNA substrate molecule of (d) comprises a library of DNA substrate molecules.

159. A method for evolving a DNA polymerase with an error rate greater than that of wild type DNA polymerase comprising:

(a) providing a library of mutant DNA substrate molecules encoding mutant DNA polymerase in a host cell comprising an indicator gene having a revertible mutation, wherein the indicator gene is replicated by the mutant DNA polymerase;

(b) screening the products of (a) for revertants of the indicator gene;

(c) recovering mutant DNA substrate molecules from revertants; and

(d) recovering a DNA substrate molecule encoding an evolved polymerase from the products of (c).

160. The method of claim 159, wherein the indicator gene is LacZalpha or GFP.

161. The method of claim 159 wherein the revertible mutation is a stop codon.

162. The method of claim 159, wherein the host cell comprises a mutant host DNA polymerase.

163. A method for evolving a DNA polymerase, comprising:

(a) providing a library of mutant DNA substrate molecules encoding mutant DNA polymerase, the library comprising a plasmid vector;

(b) preparing plasmid preparations and extracts of host cells transfected with the products of (a);

(c) amplifying each plasmid preparation in a PCR reaction using the mutant polymerase encoded by that plasmid, the polymerase being present in the host cell extract;

(d) recovering the PCR products of (c); and

(e) recovering a DNA substrate molecule encoding an evolved polymerase from the products of (d).

164. The method of claim 163, wherein the reaction of (c) is carried out in the presence of an organic solvent, a base analog, or inosine.

165. The method of claim 163, wherein (a) - (e) are repeated at least once.

166. An evolved polymerase produced by the method of claim 163.

167. The method of claim 163, wherein the products of (e) are subjected to mutagenesis.

168. The method of claim 167, wherein mutagenesis comprises recursive sequence recombination.

169. The method of claim 167, wherein the products of claim 167 are used in (a).

170. The method of claim 163, wherein the DNA substrate molecule of (e) comprises a library of DNA substrate molecules.

171. A method for evolving a p-nitrophenol phosphonatase from a phosphonatase encoded by a DNA substrate molecule, comprising:

(a) providing library of mutants of the DNA substrate molecule, the library comprising a plasmid expression vector;

(b) transfecting a host, wherein the host phn operon is deleted;

(c) selecting for growth of the transfectants of (b) using a p-nitrophenol phosphonatase as a substrate;

(d) recovering the DNA substrate molecules from transfectants selected from (c); and

(e) recovering a DNA substrate molecule from (d) encoding an evolved phosphonatase.

172. The method of claim 171, wherein (a) - (e) are repeated at least once.

173. The method of claim 171, wherein the phosphonatase is selected from the group consisting of beta-lactamase and alkyl phosphonatase.

174. An evolved p-nitrophenol phosphonatase produced by the method of claim 173.

175. The method of claim 171, wherein the products of (e) are subjected to mutagenesis.

176. The method of claim 175, wherein mutagenesis comprises recursive sequence recombination.

177. The method of claim 175, wherein the products of claim 175 are used in (a).

178. The method of claim 171, wherein the DNA substrate molecule of (e) comprises a library of DNA substrate molecules.

179. A method for evolving a protease encoded by a DNA substrate molecule comprising:

(a) providing library of mutants of the DNA substrate molecule, the library comprising a plasmid expression vector, wherein the DNA substrate molecule is linked to a secretory leader;

(b) transfecting a host;

(c) selecting for growth of the transfectants of (b) on a complex protein medium; and

(d) recovering a DNA substrate molecule from (c) encoding an evolved protease.

180. The method of claim 179, wherein (a) - (d) are repeated at least once.

181. An evolved subtilisin produced by the method of claim 179.

182. The method of claim 179, wherein the products of (d) are subjected to mutagenesis.

183. The method of claim 182, wherein mutagenesis comprises recursive sequence recombination.

184. The method of claim 182, wherein the products of claim 184 are used in (a).

185. The method of claim 179, wherein the DNA substrate molecule of (d) comprises a library of DNA substrate molecules.

186. The method of claim 179, wherein the protease is a subtilisin.

187. A method for screening a library of protease mutants displayed on a phage to obtain an improved protease, wherein a DNA substrate molecule encoding the protease is fused to DNA encoding a filamentous phage protein to generate a fusion protein, comprising:

(a) providing host cells expressing the fusion protein;

(b) overlaying host cells with a protein net to entrap the phage;

(c) washing the product of (b) to recover phage liberated by digestion of the protein net;

(d) recovering DNA from the product of (c); and

(e) recovering a DNA substrate from (d) encoding an improved protease.

188. The method of claim 187, wherein (a) - (e) are repeated at least once.

189. An evolved protease produced by the method of claim 187.

190. The method of claim 187, wherein the products of (e) are subjected to mutagenesis.

191. The method of claim 190, wherein mutagenesis comprises recursive sequence recombination.

192. The method of claim 190, wherein the products of claim 190 are used in (a).

193. The method of claim 187, wherein the DNA substrate molecule of (e) comprises a library of DNA substrate molecules.

194. A method for screening a library of protease mutants to obtain an improved protease, the method comprising:

(a) providing a library of peptide substrates, the peptide substrate comprising a fluorophore and a fluorescence quencher;

(b) screening the library of protease mutants for ability to cleave the peptide substrates, wherein fluorescence is measured; and

(c) recovering DNA encoding at least one protease mutant from (b).

195. A method for evolving an alpha interferon gene comprising:

(a) providing a library of mutant alpha interferon genes, the library comprising a filamentous phage vector;

(b) stimulating cells comprising a reporter construct, the reporter construct comprising a reporter gene under control of an interferon responsive promoter, and wherein the reporter gene is GFP;

(c) separating the cells expressing GFP by FACS;

(d) recovering phage from the product of (c); and

(e) recovering an evolved interferon gene from the product of (d).

196. The method of claim 195, wherein the interferon responsive promoter is an MHC I promoter.

197. The method of claim 195, wherein (a) - (e) are repeated at least once.

198. An evolved interferon produced by the method of claim 195.

199. The method of claim 195, wherein the products of (e) are subjected to mutagenesis.

200. The method of claim 199, wherein mutagenesis comprises recursive sequence recombination.

201. The method of claim 199, wherein the products of claim 199 are used in (a).

202. The method of claim 195, wherein the evolved interferon gene of (e) comprises a library of genes.

203. A method for screening a library of mutants of a DNA substrate encoding a protein for an evolved DNA substrate, comprising:

(a) providing a library of mutants, the library comprising an expression vector;

(b) transfecting a mammalian host cell with the library of (a), wherein mutant protein is expressed on the surface of the cell;

(c) screening or selecting the products of (b) with a ligand for the protein;

(d) recovering DNA encoding mutant protein from the products of (c); and

(e) recovering an evolved DNA substrate from the products of (d).

204. The method of claim 203, wherein the ligand is an antibody.

205. The method of claim 203, wherein the ligand is a substrate and the protein is an enzyme.

206. The method of claim 203, wherein the expression vector comprises an SV40 origin and the host cell is a Cos cell.

207. The method of claim 203, wherein the mutant protein is expressed transiently.

208. The method of claim 203, wherein the host cell further comprises SV40 large T antigen.

209. The method of claim 203, wherein the protein is an antibody.

210. The method of claim 203, wherein (a) - (e) are repeated at least once.

211. The method of claim 203, wherein the DNA substrate molecule encodes all or part of a protein selected from Table I.

212. An evolved protein produced by the method of claim 203.

213. The method of claim 203, wherein the products of (e) are subjected to mutagenesis.

214. The method of claim 213, wherein mutagenesis comprises recursive sequence recombination.

215. The method of claim 213, wherein the products of claim 213 are used in (a).

216. The method of claim 203, wherein the DNA substrate molecule of (e) comprises a library of DNA substrate molecules.

217. A method for evolving a DNA substrate molecule encoding an interferon alpha, comprising:

(a) providing a library of mutant alpha interferon genes, the library comprising an expression vector wherein the alpha interferon genes are expressed under the control of an inducible promoter;

(b) transfecting host cells with the library of (a);

(c) contacting the product of (b) with a virus;

(d) recovering DNA encoding a mutant alpha interferon from host cells surviving step (c); and

(e) recovering an evolved interferon gene from the product of (d).

218. The method of claim 217, wherein the promoter is a metallothionein promoter.

219. The method of claim 217, wherein the virus is HIV.

220. The method of claim 217, wherein the virus further comprises a conditionally lethal gene.

221. The method of claim 217, wherein the conditionally lethal gene is thymidine kinase.

222. The method of claim 217, wherein the transfected cells are exposed to conditionally lethal selective conditions.

223. The method of claim 217, wherein (a) - (e) are repeated at least once.

224. An evolved interferon alpha produced by the method of claim 217.

225. The method of claim 217, wherein the products of (e) are subjected to mutagenesis.

226. The method of claim 225, wherein mutagenesis comprises recursive sequence recombination.

227. The method of claim 225, wherein the products of claim 218 are used in (a).

228. The method of claim 217, wherein the DNA substrate molecule of (e) comprises a library of DNA substrate molecules.

229. A method for evolving the serum stability or circulation half-life of a protein encoded by a DNA substrate molecule, the DNA substrate molecule comprising a fusion of a DNA sequence encoding the protein with a DNA sequence encoding a filamentous phage protein to generate a fusion protein, the method comprising:

(a) providing a host cell expressing a library of mutants of the fusion protein;

(b) affinity purifying the mutants with a ligand for the protein, wherein the ligand is a human serum protein, tissue specific protein, or receptor;

(c) recovering DNA encoding a mutant protein from the affinity selected mutants of (b); and

(d) recovering an evolved gene encoding the protein from the product of (c).

230. The method of claim 229, wherein the serum protein is serum albumin, immunoglobulin, lipoprotein, haptoglobin, fibrinogen, transferrin, alpha-1 anti-trypsin, alpha -2 macroglobulin, or an interferon.

231. The method of claim 229, wherein the DNA sequence encoding the filamentous phage protein comprises a phage.

232. The method of claim 229, wherein the DNA sequence encoding the filamentous phage protein comprises a phagemid.

233. The method of claim 229, wherein the products of step (a) are derivitized with a half-life extending moiety.

234. The method of claim 229, wherein the moiety is polyethylene glycol.

235. The method of claim 229, wherein the DNA substrate molecule comprises a fusion of nucleic acid encoding the protein with nucleic acid encoding an epitope tag.

236. The method of claim 235, wherein the products of (a) are contacted with a protease prior to (b).

237. The method of claim 235, wherein the ligand is an antibody specific for the epitope tag.

238. The method of claim 229, wherein the protein is selected from Table I.

239. The method of claim 229, wherein the products of (a) are subjected to heat, metal ions, non-physiological pH, lyophilization, or freeze-thawing before (b).

240. The method of claim 229, wherein (a) - (e) are repeated at least once.

241. An evolved alpha interferon produced by the method of claim 229.

242. The method of claim 229, wherein the products of (d) are subjected to mutagenesis.

243. The method of claim 242, wherein mutagenesis comprises recursive sequence recombination.

244. The method of claim 242, wherein the products of claim 242 are used in (a).

245. The method of claim 229, wherein the evolved gene of (d) comprises a library of DNA substrate molecules.

246. A method for evolving a protein having at least two subunits, comprising:

(a) providing a library of mutant DNA substrate molecules for each subunit;

(b) recombining the libraries into a library of single chain constructs of the protein, the single chain construct comprising a DNA substrate molecule encoding each subunit sequence, the subunit sequence being linked by a linker at a nucleic acid sequence encoding the amino terminus of one subunit to a nucleic acid sequence encoding the carboxy terminus of a second subunit;

(c) screening or selecting the products of (b),

(d) recovering recombinant single chain construct DNA substrate molecules from the products of (c);

(e) subjecting the products of (d) to mutagenesis; and

(f) recovering an evolved single chain construct DNA substrate molecule from (e).

247. The method of claim 246, wherein the products of (b) are displayed on a phage.

248. The method of claim 246, wherein the protein is selected from Table I.

249. The method of claim 246, wherein (a) - (f) are repeated at least once.

250. An evolved protein produced by the method of claim 246.

251. The method of claim 246, wherein the products of (f) are subjected to mutagenesis.

252. The method of claim 246, wherein mutagenesis comprises recursive sequence recombination.

253. The method of claim 246, wherein the products of claim 246 are used in (a).

254. The method of claim 246, wherein the evolved DNA substrate molecule of (f) comprises a library of DNA substrate molecules.

255. A method for evolving the coupling of a mammalian 7-transmembrane receptor to a yeast signal transduction pathway, comprising:

(a) expressing a library of mammalian G alpha protein mutants in a host yeast cell, wherein the host cell expresses the mammalian 7-transmembrane receptor and a reporter gene, the receptor gene geing expressed under control of a yeast pheromone responsive promoter;

(b) screening or selecting the products of (a) for expression of the reporter gene in the presence of a ligand for the 7-transmembrance receptor; and

(c) recovering DNA encoding an evolved G alpha protein mutant from screened or selected products of (b).

256. The method of claim 255, wherein the products of (c) are subjected to mutagenesis.

257. The method of claim 256, wherein mutagenesis comprises recursive sequence recombination.

258. The method of claim 255, wherein the products of claim 255 are used in (a).

259. The method of claim 255, wherein (a) - (c) are repeated at least once.

260. An evolved G alpha protein produced by the method of claim 255.

261. The method of claim 255, wherein the reporter gene is luciferase.

262. The method of claim 255, wherein the pheromone responsive promoter is positively regulated by GAL4 and wherein GAL4 is expressed under the control of a pheromone sensitive, GAL4 enhanced promoter.

263. A method for recombining at least a first and second DNA substrate molecule, comprising:

(a) transfecting a host cell with at least a first and second DNA substrate molecule wherein the at least a first and second DNA substrate molecules are recombined in the host cell;

(b) screening or selecting the products of (a) for a desired property; and

(c) recovering recombinant DNA substrate molecules from (b).

264. The method of claim 263, wherein the products of (c) are subjected to mutagenesis.

265. The method of claim 264, wherein the mutagenesis comprises recursive sequence recombination.

266. The method of claim 263, wherein (a) - (c) are repeated at least once.

267. The method of claim 263, wherein the products of claim 263 are used in (a).

268. A method for evolving a DNA substrate sequence encoding a protein of interest, wherein the DNA substrate comprises a vector, the vector comprising single-stranded DNA, the method comprising:

(a) providing single-stranded vector DNA and a library of mutants of the DNA substrate sequence;

(b) annealing denatured double-stranded DNA from the library of (a) to the single stranded vector DNA of (a);

(c) transforming the products of (b) into a host;

(d) screening the product of (c) for a desired property; and

(e) recovering evolved DNA substrate DNA from the products of (d).

269. The method of claim 268, wherein the product of (e) is subjected to mutagenesis.

270. The method of claim 269, wherein mutagenesis comprises recursive sequence recombination.

271. The method of claim 269, wherein the product of claim 269 is used in (a).

272. The method of claim 268, wherein the host is a mutS host.

273. The method of claim 268, wherein the vector is a phagemid.

274. The method of claim 268, wherein (a) - (e) are repeated at least once.

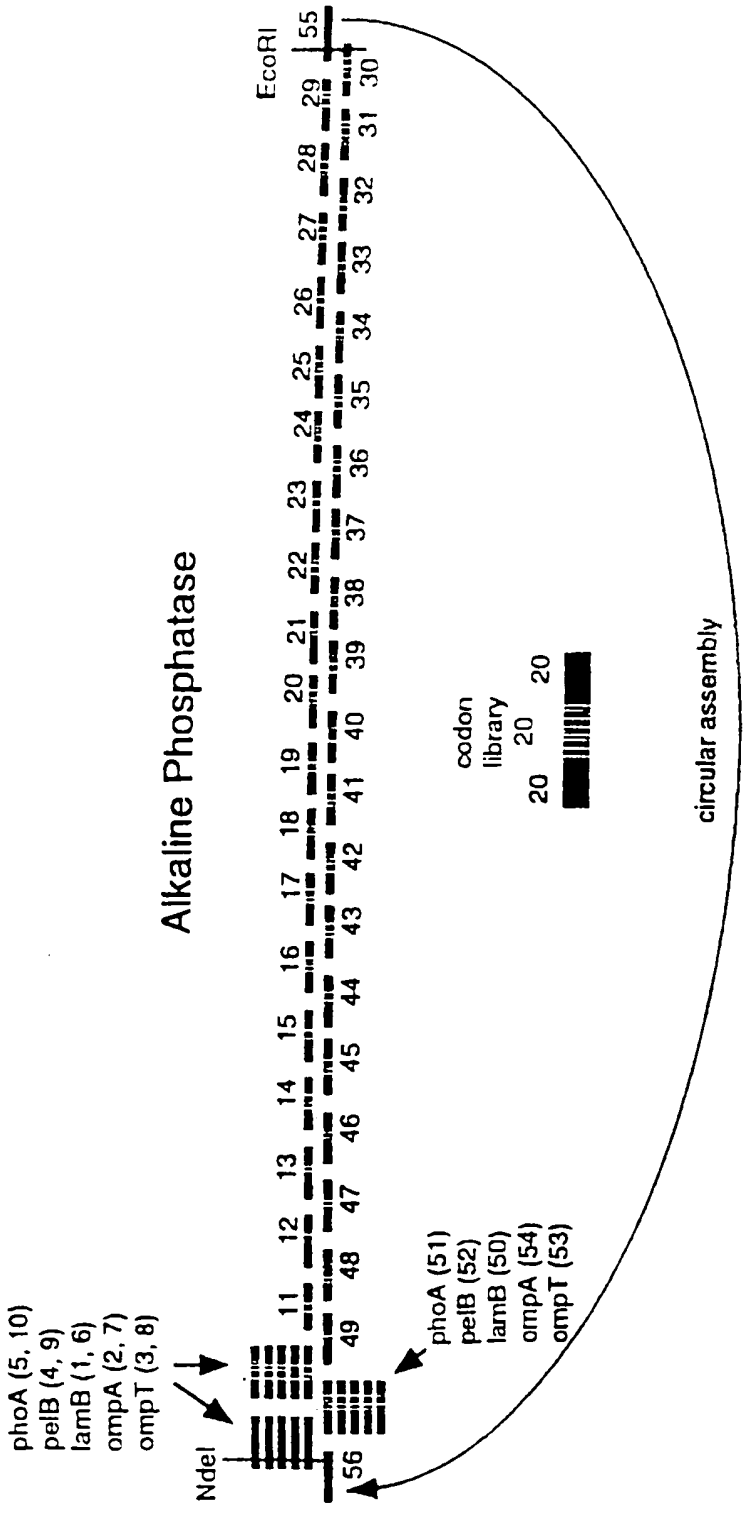

Alkaline Phosphatase
EcoRI
NdeI
phoA (5, 10)
pelB (4, 9)
lamB (1, 6)
ompA (2, 7)
ompT (3, 8)
phoA (51)
pelB (52)
lamB (50)
ompA (54)
ompT (53)
codon
library
20
20
20
circular assembly

Figure 1

Interferon Figures

Protein sequences of interferon alphas to be shuffled

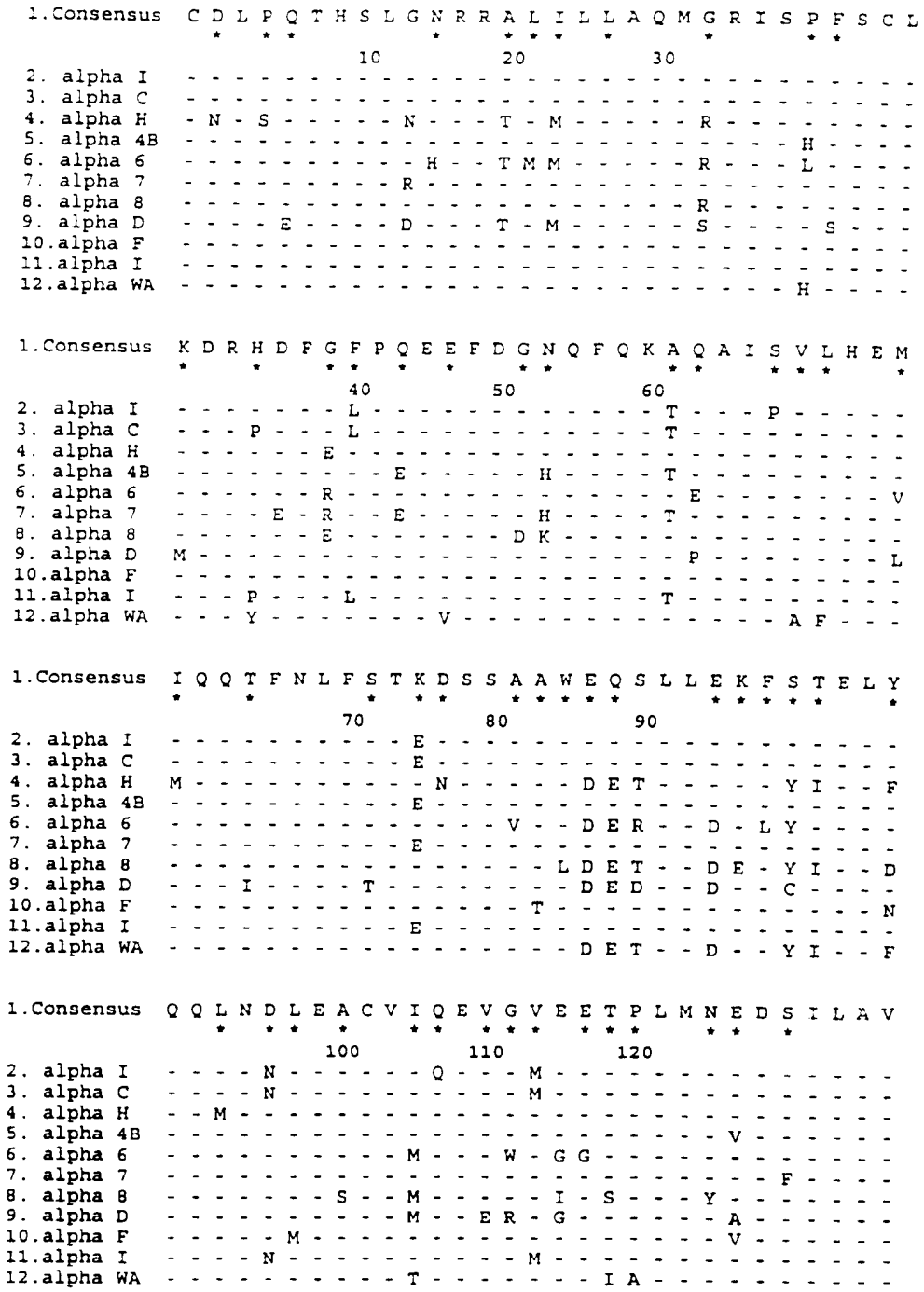


Figure 2 Page 1 of 7

```
1.Consensus  R K Y F Q R I T L Y L T E K K Y S P C A W E V V R A E I M R
                *     *           * *         *
                                130         140         150
2. alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
3. alpha C   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
4. alpha H   K - - - - - - - - - - M - - - - - - - - - - - - - - - - - -
5. alpha 4B  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
6. alpha 6   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
7. alpha 7   - - - - - - - - - - M - - - - - - - - - - - - - - - - - - -
8. alpha 8   - - - - - - - - - - - - - - - - - - S - - - - - - - - - - -
9. alpha D   K - - - R - - - - - - - - - - - - - - - - - - - - - - - - -
10.alpha F   K - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
11.alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
12.alpha WA  - - - - - - - - - - M G - - - - - - - - - - - - - - - - - -

1.Consensus  S L S F S T N L Q K R L R R K D
               *   *   * * * * * *   * *
                                160
2. alpha I   - - - - - - - - - - - - - - - -
3. alpha C   - - - - - - - - - - I - - - - -
4. alpha H   - - - - - - - - - - - - - - - -
5. alpha 4B  - - - - - - - - - - - - - - - -
6. alpha 6   - F - S - R - - - E - - - - - E
7. alpha 7   - F - - - - - - K - G - - - - -
8. alpha 8   - F - L - I - - - - - K S - E
9. alpha D   - - - L - - - - - E - - - - - E
10.alpha F   - F - L - K I F - E - - - - - E
11.alpha I   - - - - - - - - - - I - - - - -
12.alpha WA  - F - - - - - - - - G - - - - -
```

Figure 2 Page 2 of 7

(3/9)

SUBSTITUTE SHEET (RULE 26)

DNA sequences of interferon alphas to be shuffled

```
1.Consensus  T G T G A T C T G C C T C A G A C C C A C A G C C T G G G T

                          10          20          30
2. alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
3. alpha C   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
4. alpha H   - - - A - - - - - T - - - - A - - - - - - - - - - - - A A -
5. alpha 4B  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
6. alpha 6   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
7. alpha 7   - - - - - - - - - - - - - - - - - - - - - - - - - - - C - -
8. alpha 8   - - - - - - - - - - - - - - - - - - T - - - - - - - - - - -
9. alpha D   - - - - - - - - C - - - G - - - - - - - - - - - - - - - A -
10.alpha F   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
11.alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
12.alpha WA  - - - - - - - - - - - - - - - - - - T - - - - - - - - - - -

1.Consensus  A A T A G G A G G G C C T T G A T A C T C C T G G C A C A A

                          40          50          60
2. alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
3. alpha C   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
4. alpha H   - - C - - - - - - A - T - - - - - G - - - A - - - - - - - -
5. alpha 4B  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
6. alpha 6   C - C - - - - - - A - - A - - - - G - - - - - - - - - - - -
7. alpha 7   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
8. alpha 8   - - C - - - - - - - - - - - - - - - - - - - - - - - - - - -
9. alpha D   - - C - - - - - - A - - - - - - - G - - - - - - - - - - - -
10.alpha F   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
11.alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
12.alpha WA  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

1.Consensus  A T G G G A A G A A T C T C T C C T T T C T C C T G C C T G

                          70          80          90
2. alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
3. alpha C   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
4. alpha H   - - - A - G - - - - - - - - - - - - - - - - - - - - - - - -
5. alpha 4B  - - - - - - - - - - - - - - - - - - A - - - - - - - - - - -
6. alpha 6   - - - A - G - - - - - - - - - - - T - - - - - - - - T - - -
7. alpha 7   - - - - - - - - - - - - - - - - - - - - - - - - - - - T - -
8. alpha 8   - - - C - - - - - - - - - - - - - - - - - - - - - - - - - -
9. alpha D   - - - A - C - - - - - - - - - - - - - C - - - - - - T - - -
10.alpha F   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
11.alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
12.alpha WA  - - - - - - - - - - - - - - - - - - A - - - - - - - - - - -

1.Consensus  A A G G A C A G A C A T G A C T T T G G A T T T C C C C A G  <-

                         100         110         120
2. alpha I   - - - - - - - - - - - - - - - - - - - - - - C - - - - - - - -
3. alpha C   - - - - - - - - - - C - - - - - - - - - - - C - - - - - - - -
4. alpha H   - - - - - - - - - - - - - - - - - - - - - A - - - - - - - - -
5. alpha 4B  - - - - - - - - - - - - - - - - - T - - C - - - - - C - - - G - -
6. alpha 6   - - - - - - - - - - - - - - - - - - - - C A - - - - - - - - - -
7. alpha 7   - - - - - - - - - - - - - - - - - A - - C A - - - - C - - A G - -
8. alpha 8   - - - - - - - - - - - - - - - - - - - - - - A - - - C - - - - - -
9. alpha D   - T - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
10.alpha F   - - - - - - - - - - - - - - - - - - - - - - - - - - C - - - - - A
11.alpha I   - - - - - - - - - - C - - - - - - - - - - - C - - - - - - - - -
12.alpha WA  - - - - - - - - - - T - - - - - T - - C - - - - - C - - - - - -
```

Figure 2 Page 3 of 7

```
1.Consensus  G A G G A G T T T G A T G G C A A C C A G T T C C A G A A G

                          130         140         150
2. alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
3. alpha C   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
4. alpha H   - - - - - A - - - - - - - - - - - - - - - - - - - - - - - A
5. alpha 4B  - - - - - - - - - - - - - - - C - - - - - - - - - - - - - -
6. alpha 6   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
7. alpha 7   - - - - - - - - - - - - - - - C - - - - - - - - - - - - - -
8. alpha 8   - - - - - - - - - - - - - A T - - A - - - - - - - - - - - -
9. alpha D   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
10.alpha F   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
11.alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
12.alpha WA  - - - - - T - - - - - - - - - - - - - - - - - - - - - - - -

1.Consensus  G C T C A A G C C A T C T C T G T C C T C C A T G A G A T G

                          160         170         180
2. alpha I   A - - - - - - - - - - - C - - - - - - - - - - - - - - - - -
3. alpha C   A - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
4. alpha H   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
5. alpha 4B  A - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
6. alpha 6   - - - G - - - - - - - - - - - - - - - - - - - - - - - G - -
7. alpha 7   A - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
8. alpha 8   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
9. alpha D   - - - - C - - - - - - - - - - - - - - - - - - - - - - C - -
10.alpha F   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
11.alpha I   A - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
12.alpha WA  - - - - - - - - - - - - - - - C - T - - - - - - - - - - - -

1.Consensus  A T C C A G C A G A C C T T C A A T C T C T T C A G C A C A

                          190         200         210
2. alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
3. alpha C   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
4. alpha H   - - G - - - - - - - - - - - - - - - - - - - - - - - - - - -
5. alpha 4B  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
6. alpha 6   - - T - - - - - - - - - - - - - - - - - - - - - - - - - - -
7. alpha 7   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
8. alpha 8   - - - - - - - - - - - - - - - - - C - - - - - - - - - - - -
9. alpha D   - - - - - - - - - - T - - - - - - C - - - - - - T C - - - -
10.alpha F   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
11.alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
12.alpha WA  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

1.Consensus  A A G G A C T C A T C T G C T G C T T G G G A T G A G A G C

                          220         230         240
2. alpha I   G - - - - - - - - - - - - - - - - - - - - - - A C - - - - -
3. alpha C   G - - - - - - - - - - - - - - - - - - - - - - A C - - - - -
4. alpha H   - - - A - - - - - - - - - - - - - - - - - - - - - - - - C -
5. alpha 4B  G - - - - - - - - - - - - - - - - - - - - - - A C - - - - -
6. alpha 6   - - - - - - - - - - - - - T - - - - - - - - - - - - - - - G
7. alpha 7   G - - - - - - - - - - - - - - - - - - - - - - A C - - - - -
8. alpha 8   - - - - - - - - - - - - - - - - - - T - - - - - - - C - -
9. alpha D   - - A - - T - - - - - - - - - - - - - - - - - - - - - G A -
10.alpha F   - - - - - - - - - - - - - - - A - - - - - - - A C - - - - -
11.alpha I   G - - - - - - - - - - - - - - - - - - - - - - A C - - - - -
12.alpha WA  - - - - - T - - - - - - - - - - - - - - - - - - - - - C - -
```

Figure 2 Page 4 of 7

```
1.Consensus  C T C C T A G A A A A A T T T T C C A C T G A A C T T T A C

                          250         260         270
2. alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
3. alpha C   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
4. alpha H   - - - - - - - - - - - - - - - C - A - - T - - - - - - - - T -
5. alpha 4B  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
6. alpha 6   - - T - - - - - C - - - C - C - A T - - - - - - - - - - - -
7. alpha 7   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
8. alpha 8   - - T - - - - - T G - - - - C - A - - T C - - - - - - G - -
9. alpha D   - - - - - - - - - C - - - - - C - G - - - C - - - - - - C - - -
10.alpha F   - - - - - - - - - - - - - - - - - - - - - - - - - - - - A - -
11.alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
12.alpha WA  - - - - - - - - - C - - - - - C - A - - T - - - - - - - - T -

1.Consensus  C A G C A A C T G A A T G A C C T G G A A G C C T G T G T G

                          280         290         300
2. alpha I   - - - - - - - - - - - - - - A - - - - - - - - - - A - - - - - -
3. alpha C   - - - - - - - - - - - - - - A - - - - - - - - - - A - - - - - -
4. alpha H   - - - - - - - A - - - - - - - - - - - - - - - - - - - - - - - -
5. alpha 4B  - - - - - - - - - - - - - - - - - - - - - - - - - - A - - - - - -
6. alpha 6   - - - - - - G - - - - - - - - - - - - - - - - - - - - - - - - -
7. alpha 7   - - - - - - - - - - - - - - - - - - - - - - - - - - A - - - - - -
8. alpha 8   - - - - - - G - - - - - - - - - - - - - - - - - G T - - - - - - -
9. alpha D   - - - - - - G - - - - - - - - - - T - - - - - - - - - - - - - -
10.alpha F   - - - - - - G - - - - - - - - - - A - - - - - - - - - - C - - -
11.alpha I   - - - - - - - - - - - - - - A - - - - - - - - - - A - - - - - -
12.alpha WA  - - - - - - - - - - - - - - - - - - - - A - - - - - - - - - - -

1.Consensus  A T A C A G G A G G T T G G G G T G G A A G A G A C T C C C

                          310         320         330
2. alpha I   - - - G - - - - - - - - - - - A - - - - - - - - - - - - - - -
3. alpha C   - - - - - - - - - - - - - - - A - - - - - - - - - - - - - - -
4. alpha H   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
5. alpha 4B  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
6. alpha 6   - - G - - - - - - - - - - G T - - - - - - G - - G - - - - - - -
7. alpha 7   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
8. alpha 8   - - G - - - - - A - - G - - - - - - - A T - - - - T - - - - - -
9. alpha D   - - G - - - - - - - - A G A - - - - - - G - - - A - - - - - - -
10.alpha F   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
11.alpha I   - - - - - - - - - - - - - - - A - - - - - - - - - - - - - - -
12.alpha WA  - C - - - - - - - - - - - - - - - - - - - - - - - T - G - -

1.Consensus  C T G A T G A A T G A G G A C T C C A T C C T G G C T G T G

                          340         350         360
2. alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
3. alpha C   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
4. alpha H   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
5. alpha 4B  - - - - - - - - - - T - - - - - - - - - - - - - - - - - - - -
6. alpha 6   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
7. alpha 7   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
8. alpha 8   - - - - - - - - - - - - - - - - - T - - - - - - - - - - - - -
9. alpha D   - - - - - - - T - C - C - - - - - - - - - - - - - - - - - - -
10.alpha F   - - - - - - - - - - T - - - - - - - - - - T - - - - - - - - -
11.alpha I   - - - - - - - - - - - - - - - - - - - - - T - - - - - - - - -
12.alpha WA  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
```

Figure 2 Page 5 of 7

```
1.Consensus  A G G A A A T A C T T C C A A A G A A T C A C T C T T T A T

                          370         380         390
2. alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
3. alpha C   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
4. alpha H   - A - - - - - - - - - - - - - - - - - - - - - - - - - - - -
5. alpha 4B  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
6. alpha 6   - - A - - - - - - - - - - - - - - - - - - - - - - - C - - C
7. alpha 7   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
8. alpha 8   - - - - - - - - - - - - - - - - - - - - - - - - - - A - - -
9. alpha D   - A - - - - - - - - - - - A - - - - - - - - - - - - C - - -
10.alpha F   - A - - - - - - - - - - - - - - - - - - - - - - - - - - - -
11.alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
12.alpha WA  - - - - - - - - - - - T - - - - - - - - - - - - - - - - - -


1.Consensus  C T G A C A G A G A A G A A A T A C A G C C C T T G T G C C

                          400         410         420
2. alpha I   - - A - - - - - - - - - - - - - - - - - - - - - - - - - - -
3. alpha C   - - A - - - - - - - - - - - - - - - - - - - - - - - - - - -
4. alpha H   - - - - T G - - - - - - - - - - - - - - - - - - - - - - - -
5. alpha 4B  - - A - - - - - - - - - - - - - - - - - - - - - - - - - - -
6. alpha 6   - - - - - - - - - - A - - G - - - - - - - - - - - - - - - -
7. alpha 7   - - A - T G - - - - - - - - - - - - - - - - - - - - - - - -
8. alpha 8   - - - - - - - - - - - - - - - - - - - - T - - - - - - - - -
9. alpha D   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
10.alpha F   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - T
11.alpha I   - - A - - - - - - - - - - - - - - - - - - - - - - - - - - -
12.alpha WA  - - - - T G - - - - - - - - - - - - - - - - - - - - - - - -


1.Consensus  T G G G A G G T T G T C A G A G C A G A A A T C A T G A G A

                          430         440         450
2. alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
3. alpha C   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
4. alpha H   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
5. alpha 4B  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
6. alpha 6   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
7. alpha 7   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
8. alpha 8   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
9. alpha D   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
10.alpha F   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
11.alpha I   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
12.alpha WA  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -


1.Consensus  T C C T T C T C T T T T T C A A C A A A C T T G C A A A A A

                          460         470         480
2. alpha I   - - - C - - - - - - - - - - - - - - - - - - - - - - - - - -
3. alpha C   - - T C - - - - - - - - - - - - - - - - - - - - - - - - - -
4. alpha H   - - - C - - - - - - - - - - - - - - - - - - - - - - - - - -
5. alpha 4B  - - - C - - - - G - - - - - - - - - - - - - - - - - - - - -
6. alpha 6   - - - - - - - - - C A - - - - G - - - - - - - - - - G - -
7. alpha 7   - - - - - - - - - - - - - - - - - - - - - - - - - A - - - -
8. alpha 8   - - - - - - - - - - A - - - - T C - - - - - - - - - - - -
9. alpha D   - - - C - - - - - - A - - - - - - - - - - - - - - G - -
10.alpha F   - - - - - - - - - - A - - - - A - - T T - - T - - - G - -
11.alpha I   - - T C - - - - - - - - - - - - - - - - - - - - - - - - -
12.alpha WA  - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
```

Figure 2 Page 6 of 7

```
1.Consensus  A G A T T A A G G A G G A A G G A T T G A

                              490         500
2. alpha I   - - - - - - - - - - - - - - - - - - - - -
3. alpha C   - T - - - - - - - - - - - - - - - - - - -
4. alpha H   - - - - - - - - - - - - - - - - - - - - -
5. alpha 4B  - - - - - - - - - - - - - - - - - - - - -
6. alpha 6   - - G - - - - - - - - - - - - - - A - A -
7. alpha 7   G - - - - - - - - - - - - - - - - - - - -
8. alpha 8   - - - - - G - A - - - T - - - - - A - - -
9. alpha D   - - - - - - - - - - - - - - - - - A - A -
10.alpha F   - - - - - - - - - - - - - - - - - A - - -
11.alpha I   - - - - - - - - - - - - - - - - - - - - -
12.alpha WA  G - - - - - - - A - - - - - - - - - - - -
```

Figure 2 Page 7 of 7

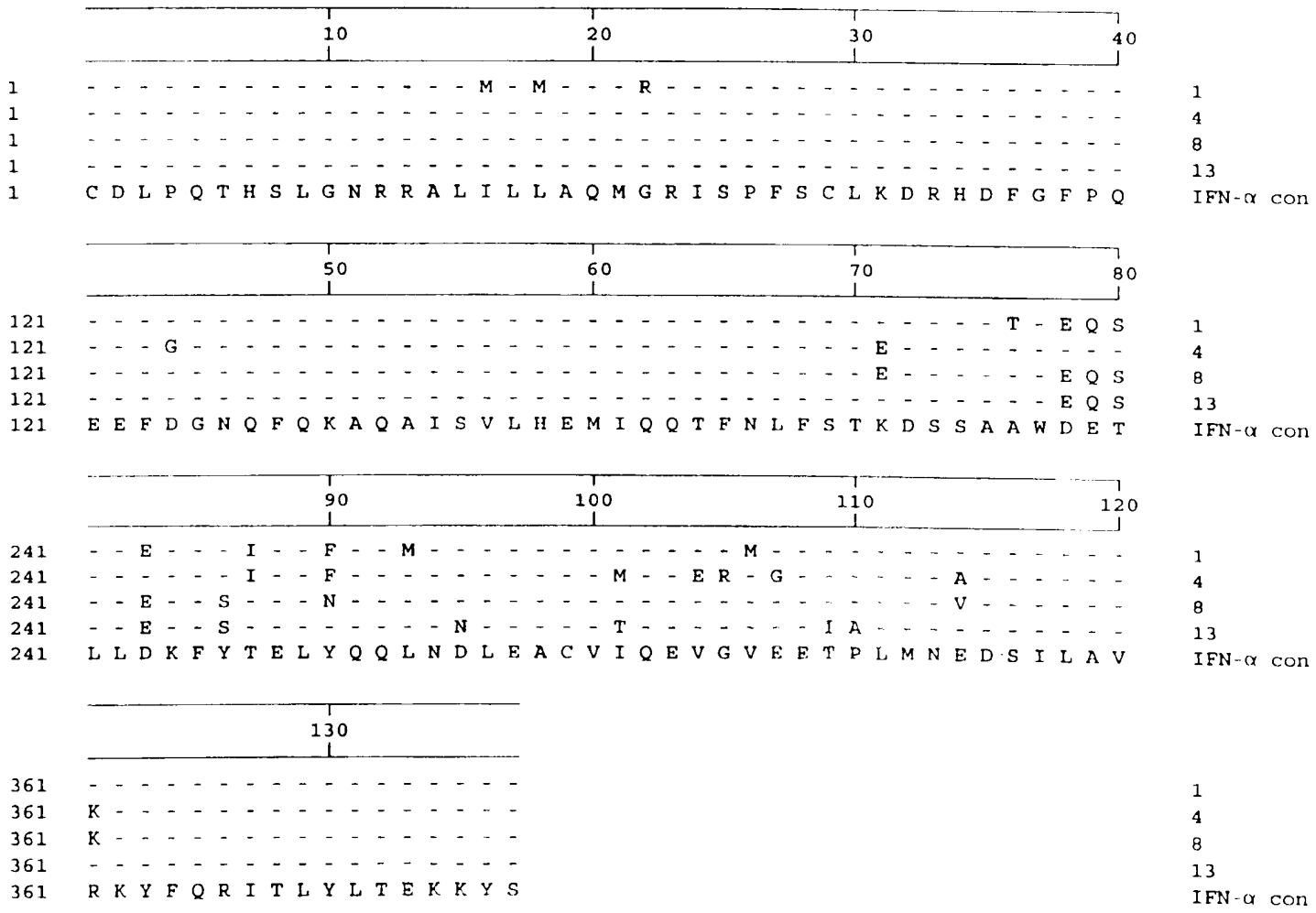

10 20 30 40
1 - - - - - - - - - - - - - - - - M - M - - - R - - - - - - - - - - - - - - - - - - 1
1 - 4
1 - 8
1 - 13
1 C D L P Q T H S L G N R R A L I L L A Q M G R I S P F S C L K D R H D F G F P Q IFN-α con
50 60 70 80
121 - T - E Q S 1
121 - - - G - E - - - - - - - - 4
121 - E - - - - - - E Q S 8
121 - E Q S 13
121 E E F D G N Q F Q K A Q A I S V L H E M I Q Q T F N L F S T K D S S A A W D E T IFN-α con
90 100 110 120
241 - - E - - - I - - F - - M - - - - - - - - - - - - - - M - - - - - - - - - - - - - 1
241 - - - - - - I - - F - - - - - - - - - - - - M - - E R - G - - - - - - A - - - - - - 4
241 - - E - - S - - - N - V - - - - - - 8
241 - - E - - S - - - - - - - - - N - - - - - T - - - - - - - - I A - - - - - - - - - - 13
241 L L D K F Y T E L Y Q Q L N D L E A C V I Q E V G V E E T P L M N E D S I L A V IFN-α con
130
361 - - - - - - - - - - - - - - - - - 1
361 K - - - - - - - - - - - - - - - - - 4
361 K - - - - - - - - - - - - - - - - - 8
361 - - - - - - - - - - - - - - - - - 13
361 R K Y F Q R I T L Y L T E K K Y S IFN-α con

Figure 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/24239

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 7.8, 69.1, 69.51, 69.7, 69.8, 70.3, 70.5, 91.2, 91.4, 91.5, 172.3, 194, 219, 222; 530/351

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG: DNA recombination, shuffling, PCR, mutagenesis, protein, evolution

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 89/06694 A1 (TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 27 July 1989, page 5, lines 8-36; page 7, lines 16-27; page 9, lines 6-16.	1-3, 14, 15 ---- 1-15
Y	GB 2,183,661 A (BALLIVET et al.) 10 June 1987, see entire document.	1-15
Y	STEMMER, W.P.C. DNA Shuffling by Random Fragmentation and Reassembly: In vitro Recombination for Molecular Evolution. Proc. Natl. Acad. Sci. USA. October 1994, Vol. 91, pages 10747-10751, see entire document.	16-37, 136-186, 246-254, 268-274
Y	JONES et al. DNA Mutagenesis and Recombination. Nature. 19 April 1990, Vol. 344, pages 793-794, see entire document.	16-37, 65-81, 136-186, 246-254, 268-274

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
30 APRIL 1998

Date of mailing of the international search report
0 3 JUN 1998

Name and mailing address of the ISA/US
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Authorized officer
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/24239

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LEVICHKIN et al. A New Approach to Construction of Hybrid Genes: Homolog Recombination Method. Mol. Biol. July-August 1995, Vol. 29, No. 5, Part 1, pages 572-577, see entire document.	16-37, 45-64, 136-186, 246-254, 268-274
Y	WO 91/00925 A1 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 24 January 1991, see entire document	38-44
Y	CHONG et al. Protein Splicing Involving the Saccharomyces cerevisiae VMA Intein. J. Biol. Chem. 06 September 1996, Vol. 271, No. 36, pages 22159-22168, especially page 22160.	45-64
Y	PRODROMOU et al. PROTOCOL: Recursive PCR: A Novel Technique for Total Gene Synthesis. Protein Eng. 1992, Vol. 5, No. 8, pages 827-829, see entire document.	82-94
Y	GATES et al. Affinity Selective Isolation of Ligands from Peptide Libraries Through Display on a lac Repressor "Headpiece Dimer". J. Mol. Biol. 1996, Vol. 255, pages 373-386, see entire document.	95-102, 113-135
Y	BENDIXEN et al. A Yeast Mating-Selection Scheme for Detection of Protein-Protein Interactions. Nus. Acids Res. 1994, Vol. 22, No. 9, pages 1778-1779, see entire document.	95-102, 113-135, 255-262
Y	US 5,565,332 A (HOOGENBOOM et al.) 15 October 1996, see entire document.	103-135, 187-202, 229-247
Y	O'NEIL et al. Phage Display: Protein Engineering by Directed Evolution. Curr. Opin. Struct. Biol. 1995, Vol. 5, pages 443-449, see entire document.	103-135, 187-202, 229-247
Y	MATTHEWS et al. Substrate Phage: Selection of Protease Substrates by Monovalent Phage Display. Science. 21 May 1993, Vol. 260, pages 1113-1117, see entire document.	187-194
Y	SWEASY et al. Detection and Characterization of Mammalian DNA polymerase β Mutants by Functional Complementation in Escherichia coli. Proc. Natl. Acad. Sci. USA. May 1993, Vol. 90, pages 4626-4630, see entire document.	150-170, 203-245
Y	SEED et al. Molecular Cloning of the CD2 Antigen, the T-Cell Erythrocyte Receptor, by a Rapid Immunoselection Procedure. Proc. Natl. Acad. Sci. May 1987, Vol. 84, pages 3365-3369, see entire document.	203-216
Y	US 5,525,486 (HONJO et al.) 11 June 1996. see entire document.	179-186

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/24239

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MANDECKI et al. FokI Method of Gene Synthesis (Recombinant DNA; oligodeoxyribonucleotide; bridge mutagenesis; cloning; gene assembly; HIV). Gene. 1988, Vol. 68, pages 101-107, see entire document.	263-267
Y	REIKOFSKI et al. Polymerase Chain Reaction (PCR) Techniques for Site-Directed Mutagenesis. Biotech. Adv. 1992, Vol. 10, pages 535-547, see entire document.	10, 32, 58, 91, 99, 115, 122, 182, 190, 213
Y	CRAMERI et al. Construction and Evolution of Antibody-Phage Libraries by DNA Shuffling. Nature Med. January 1996, Vol. 2, No. 1, pages 100-102, see entire document.	1-274
Y	CADWELL et al. Randomization of Genes by PCR Mutagenesis. PCR Meth. Appl. 1002. Vol. 2, pages 28-33, see entire document.	1-274

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/24239

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/24239

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12Q 1/68; C12P 19/34; G01N 33/53; C12N 9/12, 9/50, 9/56, 15/63; C07K 14/435

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 7.1, 7.8, 69.1, 69.51, 69.7, 69.8, 70.3, 70.5, 91.2, 91.4, 91.5, 172.3, 194, 219, 222; 530/351

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Applicant is advised that the claims lack Unity of Invention because the special technical feature, a method for "evolving" a protein was known in the prior art. See, for example, UK Patent Application, GB 2 183 661 A.

The claims are grouped according to the invention methods but include product-by-process claims to proteins.
Group I, claim(s)1-37, 45-84, 86-94, 136-149, 246-254 and 263-274, drawn to a method for recovering a recombinant DNA (no method steps involving protein expression);
Group II, claim(s) 38-44, drawn to a method for enriching a population of DNA molecules;
Group III, claim(s) 95-135 and 150-186, drawn to a non-recombinant DNA method for evolving an expressed protein;
Group IV, claim(s)187-193, drawn to a non-recombinant DNA method for screening a library of expressed protease mutants;
Group V, claim(s) 194, drawn to a non-recombinant *in vitro* method for screening protease mutants;
Group VI, claim(s)195-245 and 255-262, drawn to a non-recombinant method for evolving an expressed protein involving additional elements for expression over the method of Groups III and IV;

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The methods of Groups I-VI lack unity because the special technical feature, "evolving a protein" by recombinant DNA methods, was known in the art. The methods lack an inventive link because they are distinct in having different method steps, different modes of operation and different functions. The method of Group I lacks the step of expressing a protein which is present in the Group III and Group VI methods. The Group II method is a routinely practiced denaturing/renaturing method for enriching a DNA population and lacks the special technical feature. The method of Group IV is a specialized screen of expressed fusion protein mutants for altered activity and Group V is a routine *in vitro* screen of a library of protease mutants. These Groups also lack the special technical feature. Neither Group II nor Group IV involve a step of recovering recombinant DNA as in Group I or of expressing a protein as in Groups III and VI. The methods of Groups III and VI are distinguished by the additional elements of the Group VI methods wherein expression is coupled with a receptor or promoter which is responsive to a ligand or protein other than the evolved protein.